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(21) International Application Number: PCT/US94/10529 (22) International Filing Date: 16 September 1994 (16.09.94) (30) Priority Data: 08/123,175 17 September 1993 (17.09.93) US 08/250,975 31 May 1994 (31.05.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/250,975 (CON) Filed on 31 May 1994 (31.05.94) (71) Applicant (for all designated States except US): SMITHKLINE BEECHAM CORPORATION [US/US]; Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LEE, John, C. [US/US]; 245 Gulph Hills Road, Radnor, PA 19087 (US). ADAMS, Jerry, L. [US/US]; 611 Forest Road, Wayne, PA 19087 (US). GALLAGHER, Timothy, F. [US/US]; 255 Manor Road, Harleysville, PA 19438 (US). GREEN, David, W. [US/US]; 835 Old Lancaster Road, Bryn Mawr, PA			19010 (US). HEYS, John, Richard [US/US]; 9 Flintshire Road, Malvern, PA 19355 (US). McDONNELL, Peter, C. [US/US]; 223 B Madison Avenue, Fort Washington, PA 19034 (US). McNULTY, Dean, E. [US/US]; 229 C Fulton Street, Philadelphia, PA 19147 (US). STRICKLER, James, E. [US/US]; Apartment 307, 141 Audubon Road, Weymouth, MA 02188 (US). YOUNG, Peter, R. [US/US]; 32 Hendrickson Road, Lawrenceville, NJ 08648 (US). (74) Agents: JERVIS, Herbert, H. et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US). (81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, MD, MG, MN, MW, NO, NZ, PT, RO, RU, SD, SI, SK, TJ, TT, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BI, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DRUG BINDING PROTEIN			
(57) Abstract This invention relates to drug binding proteins, to genes encoding same and to assays and methods for screening pharmaceuticals. More specifically, this invention relates to a Cytokine Suppressive Anti-Inflammatory Drug (CSAID) binding protein, to a gene encoding same and to assays and screens useful in the evaluation and characterization of drugs of this pharmacologic class.			

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DRUG BINDING PROTEIN

Field of the Invention:

This invention relates to drug binding proteins, to genes encoding same and to assays and methods for screening pharmaceuticals. More specifically, this invention relates to Cytokine Suppressive Anti-Inflammatory Drug (CSAID) binding proteins, to genes encoding same and to assays and screens useful in the evaluation and characterization of drugs of this pharmacologic class.

Background of the Invention:

Cytokines play an important role in regulating the cellular response during inflammation and other immune functions. Of particular interest are the cytokines interleukin-1 (IL-1, α and β) and tumor necrosis factor (TNF, α and β), which are the intercellular proteins involved in the initial step of the inflammatory response cascade (Arai, *et al.*, Ann. Rev. Biochem. 59: 783-836 (1990)). Thus, there has been a substantial amount of research recently devoted to interfering with the production of IL-1 and TNF in response to an inflammatory stimulus.

One therapeutic approach involves suppressing the production of IL-1 and TNF at the level of transcription and/or translation and/or secretion. The activities associated with certain of pyridinyl imidazoles led to a class of compounds referred to as "CSAIDs", or Cytokine Suppressing Anti-Inflammatory Drugs (Figure 1). These compounds appear to arrest the expression of IL-1 and TNF predominantly at the translational level, although a lesser effect on transcription has also been observed but effects on other steps cannot be ruled out.

The pyridinyl imidazole, 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo(2,1-b)thiazole (SK&F 86002) was identified as the prototypic CSAID. The basis for its activity has been established and characterized (Lee, *et al.*, Int'l. J. Immunopharm. 10(7): 835-843 (1988); Agents and Actions 27(3/4): 277-279 (1989) and Int'l. J. Immunother. 6(1):1-12 (1990)). SAR studies (discussed herein) suggest that cytokine suppressive effect of the pyridinyl imidazoles represents a unique activity independent of their inhibitory effects on eicosanoid and leukotriene production. However, no compound of the initial series was selective for cytokine suppressive activity or was particularly potent.

Since the CSAIDs have substantial potential as novel anti-inflammatory therapeutic agents, there is significant interest in characterizing their mechanism of action at the molecular level, as well as obtaining compounds with increased selectivity and potency. Specifically, identification and characterization of the CSAID molecular target would enhance the understanding of the biochemical processes involved in inflammation and aid in the design and screening of more potent anti-inflammatory drugs. This invention discloses, *inter alia*, the purification and characterization of such CSAID binding proteins (CSBPs).

The DNAs of this invention, such as the specific sequences disclosed herein, are useful in that they encode the genetic information required for the expression of the novel CSBPs. Additionally, the sequences may be used as probes in order to isolate and identify any additional members of the CSBP family as well as forming the basis of antisense therapy for disease conditions which are characterized by atypical expression of the CSBP gene. The novel protein itself is useful directly as a therapeutic or diagnostic agent as well as a component in a screening system for compounds which are antagonists or agonists of CSAID binding activity. The protein is also useful for eliciting antibody production in heterologous species, said antibodies being useful for the aforesaid diagnostic, therapeutic and screening applications. These and additional uses for the reagents described herein will become apparent to those of ordinary skill in the art upon reading this specification.

Brief Description of the Invention:

This invention provides isolated nucleic acid molecules encoding a CSAID binding protein, including mRNAs, DNAs, cDNAs as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

This invention also provides recombinant vectors, such as cloning and expression plasmids useful as reagents in the recombinant production of CSAID binding proteins or peptides, as well as recombinant prokaryotic and/or eukaryotic host cells comprising the CSBP encoding nucleic acid sequence.

This invention also provides methods of identifying ligands capable of binding to the CSBP by measuring the binding of the ligand to be identified relative to known ligands.

This invention also provides methods for screening drugs to identify compounds which interact with and bind to the CSBP. The binding protein may be in isolated form in solution, or in immobilized form, or may be genetically

engineered to be expressed on the surface of recombinant host cells such as in phage display system or as fusion proteins. Alternatively, whole cells or cytosolic fractions comprising the CSBP may be employed in screening protocols.

Regardless of the form of the binding protein, a plurality of compounds are
5 contacted with the binding protein under conditions sufficient to form a compound/
binding protein complex and compound capable of forming, enhancing or
interfering with said complexes are detected.

This invention also provides nucleic acid probes comprising nucleic acid
molecules of sufficient length to specifically hybridize to CSAID binding protein-
10 like sequences.

This invention also provides an antisense oligonucleotide having a sequence
capable of binding with mRNAs encoding the CSBP so as to prevent the translation
of said mRNA.

This invention also provides transgenic non-human animals comprising or
15 lacking a nucleic acid molecule encoding a CSBP. Also provided are methods for
use of said transgenic animals as models for differential binding protein expression,
mutation and SAR evaluation as well as in ligand and drug screens.

This invention also provides fusion proteins comprising a CSAID binding
domain and a binding protein/ligand binding indicator domain capable of providing
20 an analytically detectable signal. Also provided are methods of screening drugs by
forming, enhancing or interfering with the detectable signal.

This invention also provides method of screening compounds to identify
those compounds which bind to a CSAID binding protein comprising: providing a
recombinant host cell expressing on the surface thereof a CSAID binding protein,
25 said protein being associated with a second component capable of providing a
detectable signal in response to the binding of a compound to said protein; contacting
a plurality of candidate compounds with said host cells under conditions sufficient to
permit binding of compounds to the binding protein; and identifying those
compounds capable of binding by detecting the signal produced by said second
30 component.

Brief Description of the Figures

Figure 1 illustrates the correlation of IC₅₀ of the pyridinyl imidazole
CSAIDs for IL-1 β biosynthesis in THP.1 cells and human monocytes. A Log-Log
35 scatter plot of ~50 compounds with regard to their IC₅₀s for inhibiting IL-1 or TNF

was generated. Regression analysis was performed and the correlation coefficient is 0.881.

Figure 2 illustrates the time dependent and reversible uptake of ^3H -Compound I in intact THP.1 cells. 2 million THP.1 cells were incubated alone (appropriate solvent control) or with radiolabeled Compound I (50 nM) in the absence (0 - 0) or presence of excess non-radioactive ligand (50 μM) Compound I (square) and Compound VIII (triangle). At various intervals, the cells were centrifuged over a 8% sucrose cushion and the cell pellet was assessed for radioactivity by scintillation counting. Saturable binding was achieved at 15 minutes.

Figure 3 illustrates the subcellular localization of binding activity. 10 million THP.1 cells were incubated with 50 nM radiolabeled Compound I for 30 minutes at 22°C. The cells were disrupted by dounce homogenization. The cell lysate was fractionated into nuclear, particulate and soluble fraction by differential centrifugation. The bulk of radioactivity was associated with the cytosolic fraction. An identical result was obtained in a binding assay using previously fractionated samples.

Figure 4 illustrates the binding isotherm and Scatchard plot analysis of Compound I binding by THP.1 cytosol. Titration of radiolabeled Compound (0 to 1 μM) in the presence of constant excess cold ligand (50 μM) was performed in the binding assay using crude THP.1 cytosol. The specific binding is saturable. Scatchard plot analysis demonstrated a K_d of 3.6 nM, B_{max} of 5 pmol/mg protein and a single site binding.

Figure 5 illustrates the specificity of the CSAID binding activity. A large number of the pyridinyl imidazole compounds spanning three different structural classes with known IC_{50} s for cytokine synthesis inhibition were tested in a competitive binding assay in which radiolabeled Compound I was used. There was a high degree of correlation between the two activities ($R=0.889$) suggesting that the binding event is a necessary step in the inhibition of cytokine production.

Figure 6 illustrates the regioselectivity of the CSAIDs. Four pairs of regioisomeric forms of the CSAIDs were tested in the bioassay and the competitive binding assay. Only one isomeric form of the respective pair was active with identical IC_{50} s in both assays.

Figure 7 illustrates that the binding of radiolabeled SB 202190 is saturable, specific and reversible. THP.1 cytosol was incubated with 50 nM radiolabeled SB Compound I for 15 minutes to allow saturable binding to equilibrate, at which time

30 μ M of the cold ligand was added and at various intervals, specific binding was determined. The binding is reversible with Compound VII and to a lesser extent, Compound XI and not at all with Compound VIII, the IC₅₀s of these compounds in the bioassay were 20 nM, 50 nM and >5 μ M respectively.

5 Figure 8 illustrates the CSAID binding activity is protease and heat sensitive. THP.1 cytosol was subjected to trypsin (100 μ g/ml) (Panel A) and heat (56°C)(Panel B) treatment. Maximum abrogation of binding activity was achieved within 2 minutes after treatment with trypsin. The binding activity was abrogated after incubation at 56°C, showed a gradual loss at 37°C and was relatively stable at 22°C
10 and 4°C.

 Figure 9 illustrates the analysis of photoaffinity labeling of CSBP by SDS-PAGE and Autoradiography. Approximately 40 μ g of protein was pre-incubated with the inhibitors listed above the gel at 10 μ M before photoaffinity labeling with ¹²⁵I Compound IV (2.5 nM). The reactions were analyzed by SDS-PAGE and
15 autoradiography as described herein.

 Figure 10 illustrates that analysis of fractions from preparative isoelectric focusing. Protein labeled with ¹²⁵I Compound IV was applied to the Rainin RF3 and analyzed as described herein.

 Figure 11 illustrates the analysis of preparative SDS-PAGE fractions by (A) SDS-PAGE and Silver Staining, and (B) Radioactivity. Fractions were analyzed as described hereinbelow.

 Figure 12 illustrates the homology of unique amino acid sequence discovered during analysis of CSBP to MAP kinase. The peptide sequence is listed below the linear representation of MAP kinase of the 15 residues; 9 identical (60%), 13
25 identical or homologous (87%).

 Figure 13 illustrates the nucleic acid sequence and amino sequence of a portion of the CSAIDs Binding Protein.

 Figure 14 illustrates the nucleic acid sequence of a second portion of the CSAIDs Binding Protein.

30 Figure 15 illustrates diagrammatically the various CSBP cDNAs described herein.

 Figure 16 illustrates the cDNA and amino acid sequence of one of the CSBP disclosed herein.

 Figure 17 illustrates the difference in nucleotide and amino acid sequence
35 between CSBP-1 and CSBP-2.

 Figure 18 illustrates a phylogenetic tree of various protein kinases.

Figure 19 illustrates the alignment of the amino acid sequences of CSBP-1 and CSBP-2 with selected members of the protein kinase family.

Figure 20 illustrates the results of expression of CSBP in *E. coli*.

Figure 21 illustrates the full length nucleic acid sequence of CSBP-1 cDNA.

5 Figure 22 illustrates the full length nucleic acid sequence of CSBP-2 cDNA.

Detailed Description of the Invention:

In further describing the present invention, the following additional terms will be employed, and are intended to be defined as indicated below.

10 An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used herein interchangeably with "immunogen."

15 The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used herein interchangeably with "antigenic determinant" or "antigenic determinant site."

20 "Fusion protein" is a protein resulting from the expression of at least two operatively-linked heterologous coding sequences. The protein comprising a CSAIDs binding protein or fragment thereof and a second unrelated peptide sequence is an example of a fusion protein.

25 A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequence is ultimately processed to produce the desired protein.

30 "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

35 A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

40 A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide when placed under the control of appropriate regulatory sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by a translation start codon (e.g., ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the expression (i.e., the transcription and translation) of a coding sequence in a host cell.

A control sequence "directs the expression" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eukaryotic cells, a stably transformed or transfected cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA or polypeptide sequences are "substantially homologous" or "substantially the same" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially homologous also refers to sequences showing identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., "Current Protocols in Mol. Biol." Vol. I & II, Wiley Interscience, Ausbel, et al. (ed.) (1992). Protein sequences that are substantially the same can be identified by proteolytic digestion, gel electrophoresis and microsequencing.

The term "functionally equivalent" with respect to CSBP intends that the amino acid sequence of the subject protein is one that will display the CSAIDs binding activity disclosed herein.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a receptor gene, the gene will usually be flanked by DNA that does not flank the gene in the genome of the source animal. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation,

alternative splicing or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

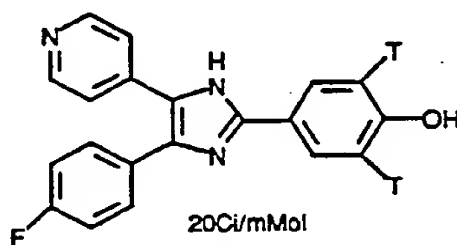
Development of Molecular Reagents:

5

Radioligand Synthesis

In order to isolate and purify the CSBP of this invention, it was first necessary to provide several labeled molecular reagents. The phenolic triaryl imidazole, Compound I, was chosen as an alternative radioligand because of its nanomolar potency and the relative ease of synthesis of the radiolabeled compound through catalytic reduction of the corresponding aryl bromide in the presence of tritium gas.

15

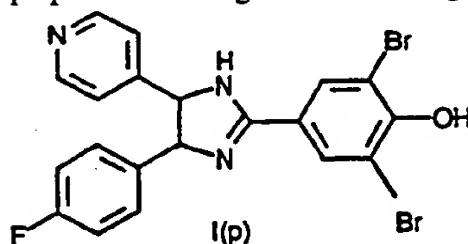


20Ci/mMol

Compound I

IL - 1 IC₅₀=0.05 μ M

Compound I was prepared according to the following reaction protocol:



2-(3,5-Dibromo-4-hydroxyphenyl)-4-(4-fluorophenyl)-5-(4-pyridyl)imidazole

MW. 489.141

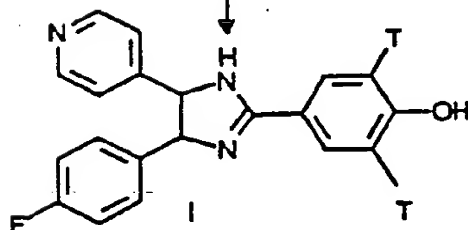
2.9 mg

Tritium gas, 5.3 Ci

Pd/C

DMF, Et₃N

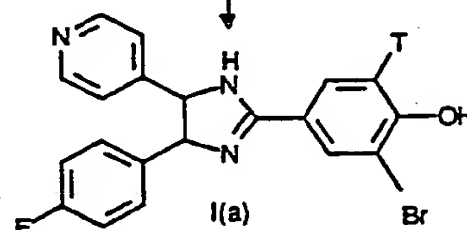
HPLC



4-(4-Fluorophenyl)-
2-(4-hydroxyphenyl)-
3,5-d₂-5-(4-
pyridyl)imidazole

331.349

48.6 mCi



2-(3-Bromo-4-hydroxy-5-t)-
4-(4-fluorophenyl)-5-(4-
pyridyl)imidazole

M.W. 410.245

10.1 mCi

HPLC

12.2 mCi

4-(4-Fluorophenyl)-
2-(4-hydroxyphenyl)-
3,5-d₂-5-(4-
pyridyl)imidazole
Compound I
9.3 mCi

Preparation of 4-(Fluorophenyl)-2-(4-hydroxyphenyl-3,5-¹²)-5-(4-pyridyl)imidazole, (Compound I).

5 A 2.9 mg (0.0059 mmol) portion of 2(3,5-Dibromo-4-hydroxyphenyl)-4-(4-fluorophenyl)-5-(4-pyridyl)imidazole, Compound I(p), was dissolved in 0.95 mL of dry DMF and 0.05 mL of triethylamine in a 2.4 mL round bottom flask equipped with a small magnetic stirring bar. A 1.7 mg portion of 5% Pd/C (Engelhard lot 28845) was added, and the flask was attached to the stainless steel tritium manifold. The mixture was degassed through four freeze-pump-thaw cycles, then tritium gas 10 (5.3 Ci, 0.091 mmol) was introduced. The reaction mixture was allowed to warm to room temperature and was stirred vigorously for 20h. The mixture was frozen in liquid nitrogen, the remaining tritium gas (2.4 Ci) was removed, and the flask was removed from the manifold. The reaction mixture was transferred, using 3 x 1 mL of methanol as rinsings, into a 10 mL round bottom flask, and the solvents were 15 removed by static vacuum transfer. A 1.5 mL portion of methanol was added to the residue, then removed by static vacuum transfer. The latter process was repeated. Finally, the residue was suspended in 1.5 mL of ethanol and filtered through a syringe-tip Millipore filter (0.45 micron), along with 3 x ca. 1 mL ethanol rinsings. The total filtrate volume was determined to be 3.9 mL, and the total radioactivity, 20 94.2 mCi. Solution was determined to be 3.9 mL, and the total radioactivity, 94.2 mCi. HPLC analysis of filtrate (Partisil 5 ODS-3, 4.6 mm I.D. x 25 cm, 1 mL/min of 70:30:01 water/acetonitrile/trifluoroacetic acid, Radiomatic Flo-One Beta radio detector with 3 mL/min of Ecoscint-H cocktail through a 0.75 mL cell) showed the presence of Compound I (R_t = 60 min. ca. 37% of total radioactivity), and a discrete 25 intermediate presumed to be the monobromo derivative Compound Ia (R_t = 11.8 min, ca. 9%).

The filtrate solution was evaporated to near dryness with a stream of nitrogen, and the residue was dissolved in about 1.2 mL of the HPLC mobile phase.

The solution was separated by HPLC as shown below, and the peaks corresponding to Compounds I and Ia and SB collected separately.

HPLC Method

Column	Altex Ultrasphere 10 mm I.D. x 25 cm
Mobile Phase	70:30:0.1 water/acetonitrile/trifluoroacetic acid
Flow Rate	5 mL/min
UV detection	210nm
Injection Volumes	0.05 - 0.4 mL
Retention Times	7.8 min Compound I 24 min Compound Ia

- 5 The pooled Compound I fractions totaled 32 mL in volume and the radioactive concentration was 1.52 mCi/mL (total 48.6 mCi). The pooled SB Compound Ia [³H] fractions (totaling 10.1 mCi) were evaporated to dryness and the residue was transferred quantitatively into a glass vial using 3.8 mL of absolute ethanol for further analysis.
- 10 An 8 mL (12.2 mCi) portion of Compound I was evaporated to dryness *in vacuo* at <35°C, then redissolved in 0.5 mL of mobile phase. The whole volume was injected into the HPLC system described above, and the appropriate peak was collected. Evaporation of the collected eluate *in vacuo* at <35°C and transfer of the yellow residue into a vial with absolute ethanol provided a solution (3.8 mL, 2.44 mCi/mL) of Compound I. The portion of this solution used for NMR analyses
- 15 was first evaporated to dryness using stream of nitrogen then taken up in CD₃OD.

Analysis of 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl-3,5- t_2)-5-(4-pyridyl)imidazole, Compound I.

Radiochemical Purity by HPLC

Method

Column	Ultrasphere Octyl, 5 μ m, 4.6 mm I.D. x 25 cm, Beckman
Mobile Phase	350:150:0.5(v/v/v) water/acetonitrile/trifluoroacetic acid
Flow Rate	1.0 mL/min
Mass detection	UV at 210 nm
Radioactivity detection	Ramona-D radioactivity flow detector
Scintillator	Tru-Count (Tru-Lab Supply Co.)
Flow rate	5.0 mL/min
Cell volume	0.75 mL
Retention time	7.7 min
<u>Result</u>	<u>98.7</u>

Radioactive Concentration by Scintillation Counting

Method

Scintillator	Ready Safe (Beckman Instruments, Inc.)
Instrument	TM Analytic model 6881
Efficiency	Automated-DPM calculation from quench curve
<u>Result</u>	<u>2.44 mCi/mL</u>

Specific Activity by Mass Spectrometry

Method

Result

CI-MS, NH ₃ reagent gas	
20.0 Ci/mmol	
³ H Distribution:	
Unlabeled	44%
Single Label	43%
Double Label	13%

³H NMR⁹

Method

Instrument	Brunker AM 400
Experiment	Proton decoupled ³ H NMR Proton non-decoupled ³ H NMR Proton non-decoupled ³ H NMR
Peak Referencing	Solvent Peak of methanol @ 3.3
Solvent	Methanol-d ₄
<u>Result</u>	Tritium is incorporated exclusively on the carbon atoms ortho to aromatic hydroxyl group

Analytical SummaryAssay

Radiochemical purity determined by HPLC

Radioactivity concentration determined by scintillation counting

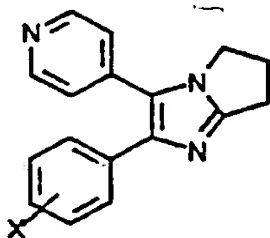
Result

98.7%

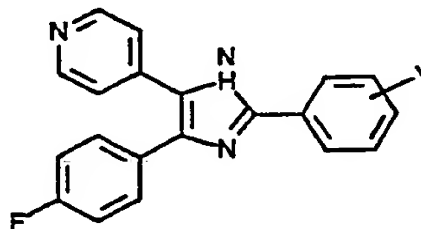
2.44 mCi/mL

Specific activity determined by mass spectrometry
 ^3H NMR20.0 Ci/mmol
agrees with the
proposed structurePhotoaffinity Radiolabeled Ligand

- 5 Additionally, a photoaffinity radiolabel was synthesized. Ideally, the radiophotoaffinity reagent should have a submicromolar binding affinity, a convenient site for the attachment of a radiolabel (preferable a gamma emitter) and allow for the positioning of the photoreactive group, (e.g. an azide) proximal to the binding site. The SAR leading to the proposal of Compound IV as the candidate for the photoaffinity reagent is illustrated in Table I below.



Formula II

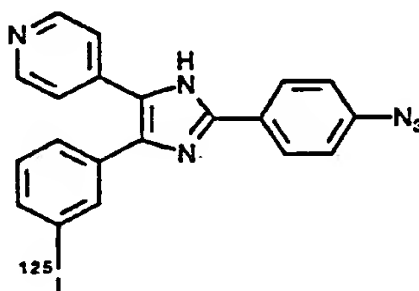


Formula III

Table I

Compound	X	BioAssay IC ₅₀ , μ M	Compound	Y	BioAssay IC ₅₀ , μ M
IIa	4-F	> 0.1	IIIa	H	0.15
IIb	4-H	0.5	IIIb	4-N ₃	0.05
IIc	4-Cl	0.05	IIIc	3-I-4-NH ₂	0.48
IId	3-Cl	0.04	IIId	4-NH ₂	0.28
IIe	2-Cl	0.25			
IIf	4-I	0.58			
IIg	3-I	0.05			

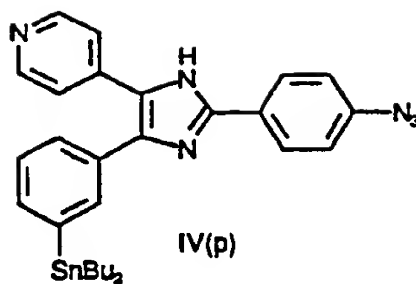
- In addition, a specific ELISA assay may also be usefully employed to
 5 determine IL-1 β and TNF α levels (see: PCT Applications US93/00674 and
 US93/00675)



Compound IV
 CSAIDs binding IC₅₀ = 0.72 μ M

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The synthesis of radioiodinated photoaffinity label, Compound IV, employed a palladium-mediated stannylation of the aryl iodide and subsequent electrophilic radioiodination, according to the following protocol.



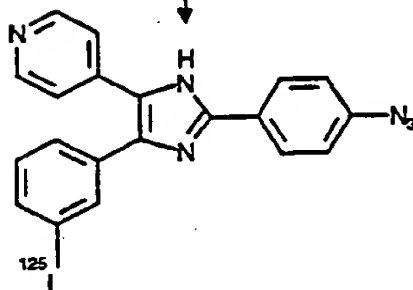
[3-[2-(4-azidophenyl)-5-(4-pyridinyl)-1H-(4-pyridinyl)-
imidazol-4-yl]phenyl]phenyl]tributylstannane

M.W.627.40

Compound IV (p)

250 µg

Na¹²⁵I
Chronic-T
3% HOAc in
EtO



4-[2-(4-azidophenyl)-5-(3-¹²⁵Iodo-phenyl)-1H-
imidazol-4-yl]pyridine

Compound IV

3.60 mCi

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Process Description

Synthesis and purification of 4-[2-(4-azidophenyl)-5-(3-¹²⁵Iodo-phenyl)-1H-imidazol-4-yl]pyridine.

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[3-[2-(4-Azidophenyl)-5-(4-pyridinyl)-1H imidazol-4-yl]phenyl]-tributylstannane, Compound IV (p) (250 µg, 0.398 µmol, was dissolved in 100 µL of 3% acetic acid in ethanol. To this solution was added 2.85 µg of chloramine-T hydrate (0.013 µmol) in 11.4 µL of water and 5.19 mCi of sodium [¹²⁵]iodine in 45 µL of 0.1 N sodium hydroxide. Another 50 µL of 3% acetic acid in ethanol was added to make

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the reaction mixture homogeneous. The reaction was stirred 60 minutes at room temperature (in the dark). The reaction was then blown to dryness under a stream of dry nitrogen and the residue partitioned between chloroform (1 mL) and saturated aqueous sodium bicarbonate (1 mL). The aqueous layer was extracted with chloroform (2 x 1 mL), the organic layers were combined and dried by passing through a pipet filled with granular sodium sulfate. The solvent was removed under stream of dry nitrogen; the residue was found to contain 4.36 mCi of iodine-125 (assayed on the Capintec dose calibrator). The aqueous layers were found to contain 310 µCi of iodine-125. The residue from the organic layer was taken up in 80 µL of HPLC mobile phase and purified on a Baker SiO₂ column, 5µm, 4.6 mm I.D. x 250 mm, eluted at 1.5 mL/min with 90:10:1 (v/v/v) hexane/isopropanol/triethylamine, with UV monitoring at 260 nm. The product fractions were combined and blown to dryness under a stream of dry nitrogen. The product was taken up in 3.0 mL of absolute ethanol. This procedure gave 3.60 mCi of Compound IV at a radiochemical purity of 99.0%, radioactive concentration of 1.20 mCi/mL and a specific activity of 1736 Ci/mmol.

Analysis of 4-[2-azidophenyl]-5-(3-iodo-¹²⁵I-phenyl)-1*H*-imidazol-4-yl]pyridine, Compound IV.

Radiochemical Purity by HPLC

Method

Column	Baker, Silica, 5 µm, 120 Å, 4.6 mm I.D. x 25 cm.
Mobile Phase	90:10:1 (v/v/v) hexane/isopropanol/triethylamine
Flow Rate	1.3 mL/min
Mass detection	UV at 260 nm
Radioactivity detection	
Detector	β-RAM radioactivity flow detector
Scintillator	Tru-Count (Tru-Lab Supply Co.)
Flow rate	5.0 mL/min
Cell size	0.8 mL
Retention time	17.0 min

Result 99.0%

Mass Concentration by HPLC

<u>Method</u>	Baker, Silica, 5 μ m, 120 A, 4.6 mm I.D. x 25 cm.
Column	
Mobile Phase	90:10:1 (v/v/v) hexane/isopropanol/triethylamine
Flow Rate	1.5 mL/min
Mass detection	UV at 260 nm
Retention time	11.2 min
<u>Result</u>	<u>99.0%</u>

Radioactive Concentration by Scintillation Counting - external standard method

<u>Method</u>	
Solvent	Ready Safe (Beckman)
Instrument	TM Analytic model 6881
Efficiency	Automated DPM calculation from quench curve
<u>Result</u>	1.2 mCi/mL

Specific Activity Derived from Mass and Radioactive Concentrations

<u>Method</u>	derived from mass and radioactive concentrations
<u>Result</u>	1736 Ci/mmol

Analytical Summary

<u>Assay</u>	<u>Result</u>
Radiochemical purity by HPLC	99.0%
Massive concentration by HPLC	0.32 μ g/mL
Radioactive concentration	1.2 mCi/mL
Specific activity derived from mass and radioactive concentrations	1736 Ci/mmol

The photoaffinity label has an IC₅₀ of 0.5-0.8 μ M in a competitive binding assay and IC₅₀ of 3 μ M in a CSAIDs bioassay.

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CSAIDs Bioassay

The biological assay employed to evaluate CSAIDs activity was the IL-1 dependent EL-4/IL2 induction assay (Simon, P. L. *et al.*, *J. Immuno. Meth.* 84: 85-94 (1985)). Briefly, Human monocytes were plated in 24-well plates in LPS-free RPMI 1640 media containing 1% human AB serum at a concentration of 10⁶

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per millilitre per well and allowed to adhere for 1 h at 37°C; non-adherent cells were removed by gentle washing. Test compounds or media were added to the cells 0 or 1 h before the addition of bacterial lipopolysaccharide (*E. coli* 001:B4; Difco, Detroit) at 10 ng/ml. The cultures were then incubated at various intervals as indicated at 37°C in a humidified 5% CO₂ atmosphere. At the end of the incubation period, culture supernatants were collected. The residual adherent monocytes were lysed in a buffer containing 0.15M octyl-glucopyranoside, 25 mM Hepes, and 0.5mM phenylmethylsulfonylfluoride in saline. Both supernatants and cell lysates were clarified by centrifugation and assayed for IL-1 activity.

IL-1 activity was measured by its ability to stimulate the secretion of IL-2 by EL-4 (ATCC TIB181) cells in the presence of A23187 ionophore. Serial dilutions of the samples were incubated with 10⁵ EL-4 cells in the presence of 2 x 10⁻⁷ M calcium ionophore A23187. After overnight incubation, 0.1 ml of a cell-free supernatant from each culture was taken and incubated with 10⁴ IL-2-dependent CTLL-20 (ATCC-TIB214) cells. Following an additional 20 hours of incubation, the cultures were pulsed with 1 µCi of tritiated thymidine for 4 h. The cells were then harvested onto glass-fibre filters and the radioactivity determined by liquid scintillation counting. All determinations of IL-1 activity were made in comparison to a standard.

CSAIDs Binding Assay

The next phase of the isolation and purification of CSBP required the development and validation of a cell-based CSAIDs binding assay. As mentioned above the early CSAID studies were conducted in human monocytes. A more convenient cell source, the human monocytic leukemia cell line, THP.1, (ATCC TIB 202) was selected and was shown to be an adequate surrogate cell source for mechanistic studies by virtue of its response to stimuli to produce IL-1 and TNF as well as a sensitivity towards CSAIDs comparable to human monocytes (Figure 1).

Radiolabeled Compound I was taken up by intact THP.1 cells in a time-dependent manner (Figure 2). The uptake of the radiolabel was rapid and reached a maximum level at 3-5 minutes at 37°C. In addition, the uptake of radiolabel was saturable and specific.

Upon subcellular fractionation of radiolabeled ligand loaded THP.1 cells, the predominant subcellular site of accumulation of the radioactivity was found to be the cytosol. (Figure 3).

A specific and reproducible CSAID binding assay was developed using soluble cytosolic fraction from THP.1 cells and radiolabeled Compound I. In brief, THP.1 cytosol was routinely prepared from cell lysate obtained by nitrogen cavitation followed by a 10 K x g low speed and a 100 K x g high speed centrifugation, the supernatant of which was designated as the cytosolic fraction. THP.1 cytosol was incubated with appropriately diluted radioligand at room temperature for a pre-determined time to allow the binding to achieve equilibrium. The sample was added to a G-10 column and eluted with 20 mM TRN, 50 μ M β -mercaptoethanol, NaN_3 . The fraction encompassing the void volume was collected and the radioactivity was assessed by liquid scintillation counting. This was determined to reflect bound radioligand since the radioactive signal was abrogated by the presence of excess cold ligand in the incubation mixture or when there was no cytosolic fraction present.

More specifically, the CSAID Binding Assay is performed as follows:

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Materials:

Incubation buffer: 20 mM Tris, 1 mM MgCl_2 , 20 μ M Hepes, 0.02% NaN_3 , store at 4°C. Elution buffer: 20 mM Tris, 50 μ M 2-mercaptoethanol, NaN_3 , store at 4°C.

20 G-10 Sephadex: add 100 g Sephadex G-10 (Pharmacia, Uppsala, Sweden) to 400 mL dd H_2O and allow to swell at room temperature for 2 hours. Decant fines and wash 3 times. Add NaN_3 and QS with dd H_2O to 500 mLs and store at 4°C.

25 Assemble Columns: Straw column, filter frit and tip (Konotes, SP 420160-000, 420162-002). Lowsorb tubes (Nunc) used in binding reaction. THP.1 cytosol spun at 15000 rpm for 5 min to clarify. THP.1 cytosol prepared by hypotonic treatment of cells and lysis by decompression in nitrogen. Nuclei and membrane fragments removed by differential centrifugation (10,000 g for 1 hour and 100,000 g for 1 hour).

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Compounds: Non-radioactive Compound I with corresponding EtOH control (dilutions made in incubation buffer) and ^3H -Compound I (dilutions in incubation buffer).

Method:

- A. Column Preparation
1. Begin 30 min before anticipated elution of reaction mixture.
 2. Add 3 mL of G-10 slurry to column for bed vol of 1.5 ml.
 3. Rinse with 7 mL elution buffer (fill to top of column)
 4. Cut columns down to size.
- B. Sample Incubation
1. 15 min incubation at 4°C.
 2. Binding reaction mixture; 100 µL cytosol, 10 µL cold Compound I or EtOH control, 10 µL ³H-Compound I (molar concentration depends on nature of study).
 3. "Free" control = 100 µL incubation buffer in lieu of cytosol preparation.
- C. Sample Elution
1. Elute at 4°C.
 2. Add total reaction volume to G-10 column.
 3. Add 400 µL elution buffer to column and discard eluate.
 4. Add 500 µL elution buffer to column, collecting eluted volume in 20 ml scintillation vial.
 5. Add 15 mL Ready Safe scintillation fluid.
 6. Vortex and count in liquid scintillation counter for 5 minutes. Include a "total input counts control" (10 µL of labeled ligand).
- D. Data Analysis
1. Plot DPMS as output in graphic form and analyze by regression analysis and "Lundon ligand binding" software for the determination of IC 50 and K_d/K_i respectively.
 2. Rank order the IC₅₀s of the tested compounds in the CSAID bioassay and compare to that generated by the CSAID binding assay and establish a correlation curve.

The binding assay was further validated by the following criteria:

- THP.1 cytosol demonstrated saturable and specific binding of radiolabeled Compound I (Figure 4).
- A substantial number of pyridinyl imidazole CSAIDs were tested in the radiolabel competitive binding assay. The rank order potency and the IC₅₀s of the compounds was highly correlative to that determined by the human monocyte bioassay (Figure 5). Furthermore, the competitive binding activity was regioselective (Figure 6). These results underline the particular usefulness of the binding assay to the cytokine suppressive effects of these compounds and is considered particularly advantageous for SAR development and providing the means to help elucidate the molecular target.
- Binding is highly specific for the pyridinyl imidazole CSAIDs. A series of non-structurally related compounds of varied pharmacological activities were tested in the competitive binding assay. They include the specific cyclooxygenase inhibitors, 5-lipoxygenase inhibitors, dual CO/LO inhibitors, PDE IV inhibitors, immunosuppressive macrolides, steroids, and others (Table II). None of the compounds tested at 100 μ M demonstrated competitive binding.

A list of non-pyridinyl imidazole CSAIDs, related anti-inflammatory or immunosuppressive compounds tested in the competitive CSAID binding assay is provided in Table II. Unless otherwise indicated, no competitive binding was observed up to 100 μ M.

TABLE II

Cyclooxygenase Inhibitors <ul style="list-style-type: none">• Indomethacin• Naproxen	Steroid <ul style="list-style-type: none">• Dexamethasone
Selective 5-Lipoxygenase Inhibitors <ul style="list-style-type: none">• Hydroxyurea class• Aminophenol class	Novel Anti-Inflammatories <ul style="list-style-type: none">• IX270• Tenidap (IC₅₀ = 139 μM)• Romazarit

5-Lipoxygenase Translocation Inhibitor • MK886	Peroxisome Proliferators • Wyeth 14643 • Clofibrate
Dual Inhibitors • Phenidone • NDGA (IC ₅₀ = 154 μ M)	AH Receptor Agonists • 3-Methylcholanthrene • β Naphthoflavone
Immunosuppressives • FK506 • Azaspirane • Rapamycin & Analogs	Miscellaneous • Tibenclast • Tetrandrine
PDE _{IV} Inhibitor • Rolipram	

Having established a cell source and a binding assay, further characterization of CSBP established that the CSAID binding is saturable, specific and reversible (Figure 7), follows a rapid on and off rate, the binding activity is sensitive to protease and heat treatment (Figure 8) and is protein concentration dependent (data not shown).

The CSAID binding activity in human monocytes is indistinguishable from that determined for THP.1 by the criteria established for the binding activity listed above.

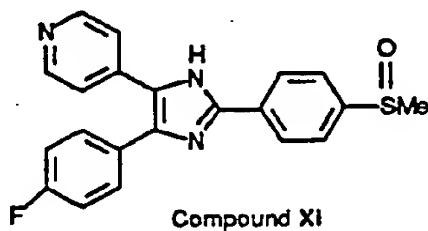
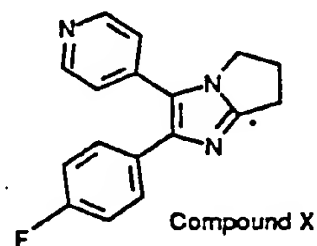
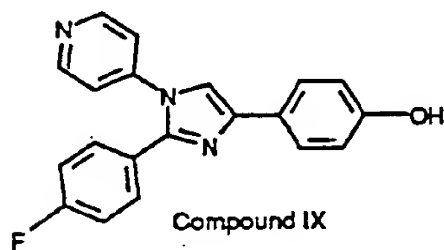
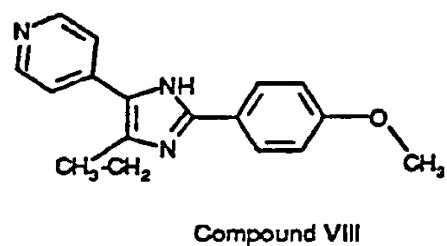
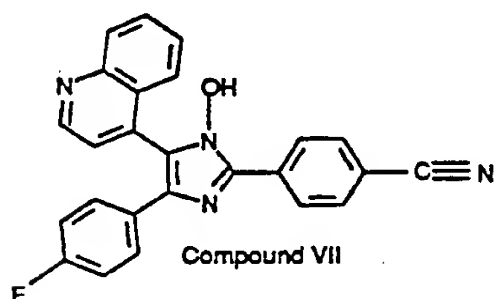
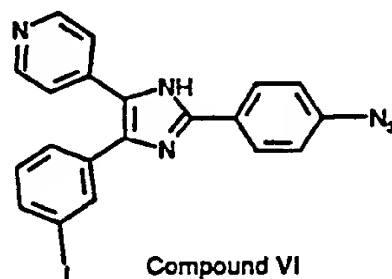
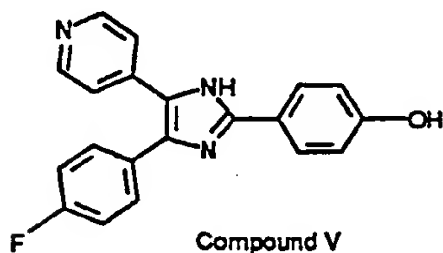
The binding is pH dependent with an optimal pH range from 5 to 8 and is independent of divalent cations and is sensitive to high salt concentration which is reversible.

Purification of CSBP

The purification of the CSBP from THP.1 cells was accomplished as follows:

Materials

The following compounds were synthesized by the methods outlined in PCT application, US93/00674 and US93/00675 both filed January 13, 1993.



The radiolabeled compounds II and IV were prepared as described above. Polyclonal and monoclonal antibodies against actin (rabbit (cat #65-096) and mouse (cat. #69-100), respectively) were purchased from ICN Biomedicals. The peptide NH₂-Ile-Thr-Ala-Ala-Gln-Ala-Leu-Ala-His-Ala-Tyr-Phe-Ala-Gln-Tyr-Cys-COOH (Seq. LD. No. 1) was synthesized by standard solid phase Fmoc chemistry (see for example: Fields, G.B., et al. Int'l. Peptide Protein Res. 35: 161-214 (1990), purified and coupled to maleimide activated keyhole limpet hemocyanin (KLH) (Pierce Chemical Co. Cat # 77105A) by conventional methods, and used to inoculate rabbits. All other chemicals were of reagent grade and unless otherwise specified, were not purchased from a particular vendor.

Growth of THP.1 Cells

THP.1 cells were grown and processed as follows:

THP. 1 cells are grown in RPMI - 1640 medium with 25 mM Hepes, 10% FBS (8% in reactors), 10mM glutamine, and .05% pluronic F-68. The cells were passed on a 3/4 days cycle with an average cell count of 2×10^6 (seeding density between 2×10^5 and 3×10^5). A high density cell recycle in shake flasks was used to scale-up the cells to the large reactors. In this process, the total volume of the shake flask was spun down and resuspended with the same volume of fresh medium. Therefore, seeding density increased with each passage, giving a higher density of cells per volume. The densities ranged from 6×10^6 to 12×10^6 .

From the shake flasks, two scale-up procedures were used to obtain the required volumes. Initially, two 80L artisan reactors (60L working volume) were used. Every five days, 50L was taken out of both reactors and harvested. The cells were then fed with an additional 50L until the total required volume was reached. Alternatively, cells were grown in a 30L artisan and used to seed the 250L Abec reactor (totaling working volume was 150L). 120L was harvested every five days and the 30L left was refed. The seeding density was between 3×10^5 and 5×10^5 . The pH for both types of reactors was controlled between 7.0 and 7.2. CO₂ was used as the controlling acid and sodium bicarbonate as the buffer. The D.O. was set at 30 percent for the Artisans reactors and 20 percent for the Abec reactor.

Preparation of THP.1 Cytosol

Cells were lysed by nitrogen cavitation in 20 mM TrisHCl pH 7.4, 1mM MgCl₂, 1mM PMSF, 1μM pepstatin A and 1μM leupeptin. Insoluble material was pelleted at 10,000 x g for 10 min and the supernatant further clarified by a 100,000

x g centrifugation for 1 h at 4°C. The supernatant from the final centrifugation was collected and is hereafter referred to as the THP.1 cytosol.

Measurement of CSAID Binding Activity

- 5 The same (typically 200 µg protein) was incubated with appropriately diluted ^3H - Compound I (50 nM) at room temperature for 60 min to allow the binding to achieve equilibrium. Free ligand was separated from bound ligand on a 1.5ml Sephadex G-10 column in 20 mM TrisHCl pH 7.4. The fraction encompassing the void volume was collected and the radioactivity was assessed by
- 10 liquid scintillation counting. Protein concentrations were determined by the bicinchoninic acid assay (Pierce).

Superose 12 Chromatography

- Approximately 100 to 250 ml of THP.1 cytosol was applied at 14.5 cm h⁻¹
- 15 to a 5 L Superose 12 column (Pharmacia; 11.5 x 50 cm) equilibrated in 10 mM NaPO₄ pH 7.0 and 150 mM NaCl at 4°C. Fractions were collected (50ml) and assayed for CSAID binding activity; a single peak of activity corresponding to an elution volume for a protein of M_r ~ 50,000 was pooled (200 to 500 ml).

Hydroxylapatite Chromatography

- The material from the Superose 12 column were applied at 30 cm h⁻¹ to a 160 ml Hydroxylapatite HA column (Cal. Biochem; 5.0 x 8.0 cm) equilibrated in 10 mM NaPO₄ pH 7.0 at room temperature. The column was eluted with a 10 to 200 mM NaPO₄ gradient over 2.5 column volumes. Fractions (30 ml) were collected
- 25 and assayed for CSAIDs binding activity. A protein peak containing approximately 60% of the CSAID binding activity applied to the column was pooled (50 to 250 ml).

Radiophotoaffinity Labeling of CSBP

- 30 The following protocol was used for about 30 ml of sample but can be adapted for larger or smaller volumes. The hydroxylapatite pool was concentrated to about 30 ml using an Amicon stir cell (YM30 membrane, 70 psi N₂). Insoluble material in the concentrate was removed by centrifugation (10,000 x g for 30 min in SS34 rotor at 4°C). The supernatant (450 mg protein) was used in the labeling
- 35 reactions, which were performed in 6-well microtiter plates (Nunc). Six reactions were carried out using the following reagents and protocol. Approx. 60 mg. of

protein (4 ml) was added to 0.25 ml buffer (10 mM NaPO₄ pH 7.0, 150 mM NaCl) and 0.25 ml 50 nM radioactive (i.e. "hot") ¹²⁵I Compound IV (final concentration of 2.5 nM, 250 µCi) in dim light and allowed to stand on ice for 10 to 15 min. The microtiter plate was exposed to > 300 nm light at a distance of 5 to 10 cm for 2 min while on ice. The reactions were chased with Compound IV (Compound VI being the "cold" (i.e. non-radioactive) form of Compound IV) as follows. A 1 mM stock of Compound VI was prepared by adding 0.3 ml 10mM Compound VI to 2.7 ml 50% ethanol in 10 mM NaPO₄ pH 7.0 and 150 mM NaCl. Compound VI (0.5 ml 1 mM) was added to each labeling reaction in dim light and allowed to stand for 10 to 15 min on ice. The reactions were exposed to light as for the radioactive labeling. Unreacted Compounds IV and VI can be removed from labeled protein by the preparative isoelectric focusing or electrophoresis steps; or for samples of smaller volume, removed by gel filtration chromatography on Sephadex G-25 (1.6 x 12 cm) in 20 mM NaPO₄ pH 7.4 and 150 mM NaCl.

15

Analytical Electrophoresis, Autoradiography and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions essentially as described by Smith B.J., Meth. in Mol. Biol., Vol I, pp. 44-57 (1984). Samples were run on 0.75 mm thickness 16 cm (4% stacking, 10 or 12% separating) or 10 cm (12% pre-cast, Jule) stab gels using the Hoefer SE 600 or Mighty Small electrophoresis systems, respectively. Protein was stained by either coomassie blue R350 (Pharmacia) or silver (Silver Stain Plus, BioRad). Molecular weight protein standards were purchased from Amersham or BioRad. For blotting, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) in 192 mM glycine/25 mM Tri pH 8.3 and 20% (v/v) methanol using a Genie electrophoretic blotter (Idea Scientific) at 15 V. Protein labeled with ¹²⁵I was visualized by autoradiography using Hyperfilm-MP (Amersham) after overnight exposures at -70°C. The membrane was blocked with 5% gelatin in 20 mM TrisHCl pH 7.5 and 500 mM NaCl before incubation with the appropriate antiserum diluted 1,000 to 5,000-fold in buffer. The antibody complexes were detected with anti-mouse or anti-rabbit immunoglobulin G (Amersham) coupled to horse radish peroxidase and visualized by luminol phosphorescence on Hyperfilm-ECL (Amersham).

30

Preparative Isoelectric Focusing

Preparative isoelectric focusing was performed using a Rainin RF3 recycling free flow focusing protein fractionator at 4°C overnight, concentrated to about 3 ml with an Amicon stir cell (YM30 membrane, 70 psi N₂), and brought to 10% glycerol and 1% ampholyte (Pharmacia Ampholine or Pharmalyte pH 4 to 6) for a final volume of about 10 ml. Before the sample was applied to the RF3, a 1% ampholyte/10% glycerol solution was pre-focused for 1 to 1.5 h (until the voltage, current, power and temperature were at baseline). The sample was injected into bubble port 14 using a needle and syringe. The system was allowed to equilibrate as for the pre-focusing before collecting 3 ml fractions. Labeled CSBP was identified by monitoring the radioactivity, and the appropriate fractions pooled.

Preparative SDS-Page

Preparative SDS-PAGE was performed using the BioRad Model 491 Preparative cell. The pooled fractions from the preparative isoelectric focusing were concentrated to 2 to 3 ml with an Amicon stir cell (YM30 membrane, 70 psi N₂). Approximately 2 to 2.5 ml of the concentrate was brought to about 3 ml in 100 mM Tris pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.01% bromophenyl blue before incubating at 100°C for 3 to 5 min. The sample was applied to the gel (2 cm 4% stacking gel, 6 cm 11% separating gel) and run at 40 mA in 192 mM glycine/25 mM Tris pH 8.3 and 0.1% SDS at room temperature. Fractions (2.5ml) were collected and assayed for radioactivity in order to identify where labeled CSBP eluted from the gel.

25 Results

Partial Purification of CSBP

A typical partial purification of CSBP from THP.1 cytosol is summarized in Table III. As indicated, the recovery of activity is 20% and the level of purification is 3-fold. This was characteristic of CSBP recovery and purification during evaluation of a number of chromatography resins (anion and cation exchange, hydrophobic interaction with (NH₄)₂SO₄, blue sepharose, heparin sepharose, etc.); the purification scheme as listed in the Table III gave the best recovery and most reproducible results. Since attempts to purify CSBP further while following CSAID binding activity resulted in poor recovery of activity, this was as far as the purification was taken before photoaffinity labeling.

Table III
Purification of CSBP from THP.1 cytosol

Sample	Activity, dpm ^a	Protein, mg	Specific Activity, dpm mg ⁻¹
THP.1 cytosol ^b	5.0×10^8	6800	7.4×10^4
Superose 12	1.6×10^8	1200	1.3×10^5
Hydroxylapatite	9.6×10^7	500	1.9×10^5

5 ^aactivity is expressed as the ³H radioactivity (disintegration per minute, dpm) collected in the CSAID binding assayed as described above and corrected for the total sample.

^bTHP.1 cytosol was prepared from starting material equivalent to approximately 10¹¹ cells.

10

Photoaffinity Labeling of CSBP

 CSBP was covalently labeled with the ¹²⁵I, aryl azide CSAID derivative Compound IV. The reaction was very specific as illustrated in Figure 9, which shows that a single protein of M_r 43,000 was labeled (the lanes labeled "None").

15 During the partial purification described above the CSAID binding activity eluted as a single peak from the Superose 12 gel filtration chromatography with a molecular weight corresponding to a protein of M_r 45,000 to 50,000. Collectively, these two analyses indicate the CSBP is a single-chain, or "monomeric" protein of M_r 43,000.

 Figure 9 also illustrates the specificity of the labeling. In the middle lanes of the gel, protein was preincubated with a non-radioactive CSAID (10 μM) before the photoaffinity labeling with ¹²⁵I Compound IV (2.5 nM). The extent to which each CSAID competed with the photoaffinity label correlates well with its potency in a cellular assay. That is, the more potent the compound is in its ability to suppress IL-1 production in human monocytes, the more effectively it prevented photaffinity labeling of the CSBP. Thus, CSBP is the protein labeled with Compound IV.

25

Purification of Labeled CSBP

 In order to identify CSBP by its amino acid sequence, the labeled protein was further purified from the partially-purified CSBP used for photaffinity labeling.

30 The strategy to accomplish this was preparative isoelectric focusing, preparative SDS-PAGE and reversed-phase HPLC. The results of the preparative isoelectric

focusing are shown in Figure 10. The isoelectric point of the labeled protein corresponded to a pH of about 4.5. Western analysis indicated that some, but not all, of the actin was removed by this procedure. In addition, almost 70% of the protein applied eluted with the labeled protein (50% recovery of radioactivity). This was also demonstrated by SDS-PAGE and silver staining analysis (data not shown). Thus, for this application preparative isoelectric focusing did not provide a substantial purification of the desired protein.

The most substantial purification of labeled CSBP was obtained by preparative SDS-PAGE. The material pooled from preparative isoelectric focusing was applied to a gel using the BioRad Model 491 Preparative Cell. As illustrated in Figure 11, the radioactive fraction corresponding to a protein of about 43 kDa (fraction 56) has at least 90% of the non-radioactive protein removed by this procedure. In addition, unincorporated label is also removed.

15 Characterization of CSBP

After preparative SDS-PAGE, labeled CSBP was applied to reversed-phase HPLC, where a protein peak coeluting with the radioactivity was collected. Comparison of the protein concentration (determined by amino acid analysis) to the specific radioactivity of the sample demonstrated that only 10% of this protein was labeled (assuming a protein M_r of 43,000). N-terminal sequence analysis identified actin sequences corresponding to 30 to 40 amino acids downstream from the expected amino terminus. Internal sequence analysis following fragmentation with trypsin or CNBr generated approximately 90% actin sequences, but about 10% of the peptides gave unique sequences. One of the sequences from the tryptic digest had strong (85%) homology, but was not identical, to a C-terminal sequence found in a family of Ser/Thr protein kinases known as the mitogen-activated (MAP) kinases (Figure 12; See also: Ray, L.B. & Sturgill, T.W., Proc. Nat'l. Acad. Sci. (USA), 85:3753-3757 (1988)).

A peptide based on the sequence with homology to the MAP kinases was synthesized and used to inoculate rabbits for the production of antisera. Western analysis and autoradiography of labeled THP.1 cytosol 2-D gels demonstrated that 1) antibodies against actin or MAP kinases did recognize proteins on the blot, but not the radiolabeled protein; 2) the antibody prepared from the tryptic peptide recognized the radiolabeled protein. Thus, CSBP appears to have homology to, but is distinct from, the MAP kinases. Given the role of kinases in regulating translation (Pelech and Sanghera, Science 257:1355-66 (1992)) and the effect of

CSAIDs on IL-1 and TNF translation, a kinase is not inconsistent as the molecular target for CSAIDs.

Isolation and Characterization of

5 the CSBP gene:

This invention provides an isolated nucleic acid molecule encoding the human CSBP. Two amino-terminal peptide sequences were obtained from the protein fraction comigrating with the radioactive photoaffinity probe. One of these

10 itself radioactive, and had the sequence:

ILE THR ALA ALA GLN ALA LEU ALA HIS ALA TYR PHE ALA GLN TYR
(Seq. I.D. No. 1)

15 The second was obtained from an 8KDa cyanogen bromide fragment associated with radioactivity and had the sequence:

XXX (GLN) LEU LEU ASN ASN ILE (VAL/PHE) LYS (PHE) GLN LYS LEU
THR (Seq. I.D. No. 2)

20 where () represents an uncertain assignment and / represents an uncertainty between two amino acids. XXX is an unknown amino acid. A search of Genbank indicated that peptide sequence I.D. No. 1 was homologous to the MAP kinase family of protein kinases, whereas peptide sequence I.D. No. 2 was unique. Based on these two sequences, two degenerate oligonucleotide DNA probes were
25 synthesized using the genetic code to reverse translate the protein sequences, and tables of mammalian cell codon preferences (Grantham, R. et al., Nucl. Acid Res. 9: (1981)).

1. GCYCAYGCTAYTTYGCYCARTA (Seq. I.D. No. 3) and

2. AAYAAYATYKTBAARTTYCAAA (Seq. I.D. No. 4)

30 where Y = C or T

R = A or G

K = G or T

B = G, C or T

Hence the two mixed oligonucleotides consist of 128 and 384 unique sequences
35 respectively. A cDNA library made from human monocytes treated with GM-CSF (Livi, G.P. et al., Mol. Cell Biol. 10: 2678-86 (1990) in the commercial vector

λZAP (Stratagene) which was screened at low stringency by hybridization to a 50:50 combination of the two synthetic oligonucleotide mixtures labeled with γ-32 P ATP. Labeling of the oligonucleotides followed published methods (Current Protocols in Molecular Biology), typically labeling 3 μg of mixed oligonucleotide with 250 μCi γ-32 P ATP and using all of this in a 250 μl hybridization volume. The manufacturer's recommended conditions for plating and lifting phage were followed (see Stratagene λZAP protocol, Stratagene, La Jolla, Ca.) using the BB4 host strain. One additional step was to prewash the filterlifts at 65°C in 2xSSPE/0.1%SDS twice for 30 min. prior to prehybridization to remove bacterial debris.

Subsequently, prehybridization and hybridization with the labeled oligonucleotide probes were performed at 37°C for 24-72h in 6xSSPE, 5xDenhardt's solution, 0.1% SDS and 100 μg/ml phenol/chloroform extracted yeast tRNA. (20xSSPE is 3M NaCl, 0.2M NaH₂PO₄, 0.02M EDTA pH7.4. 50 x Denhardt's solution is 10 g polyvinylpyrrolidone (MW 40,000), 10g Bovine serum albumin and 10 g Ficoll 400 per liter H₂O.

After hybridization the filters were washed twice under each of the following conditions.

1. 6xSSPE, 0.1% SDS, room temp, 10-15 min.
2. 6xSSPE, 0.1% SDS, 37°C, 10-15 min.
3. 3M tetramethylammonium chloride solution (500g Me₄NCl, 1.38 liter H₂O, 73 ml 1M tris pH 8.0, 5.8ml 0.5M EDTA, 7.3ml; 20% SDS filtered through 0.45 μm filter), 37°C, 30 min (see: Proc. Natl. Acad. Sci. USA 82: 1585-1588 (1985) for a description of this technique).

Filters were exposed to Kodak film for 3-5 days in the presence of intensifying screens, and overlapping positives in duplicate filters picked and cycled through the same procedure until pure plaques obtained.

Phage was excised with M13 helper phage R408 in the recA⁻ *E. coli* host XL-1 blue according to manufacturers procedures (Stratagene).

After two subsequent rounds of replating and hybridization of positively hybridizing plaques using just the oligonucleotide mixture #1, a single homogeneous phage was obtained which hybridized in a Southern blot with the oligonucleotide #1 (Seq. I.D. No. 3) but not with oligonucleotide #2 (Seq. I.D. No. 4). Sequencing of the DNA insert of this phage revealed an open reading frame at

one end which encoded part of the No. 2 unique peptide sequence. I.D. No. 2 above. The amino sequence so encoded was:

Asn Ile Val Lys Cys Gln Lys Leu Thr. (Seq. I.D. N . 5).

The rest of the open reading frame (Figure 13) Seq. I.D. No. 6 and 7 was
5 homologous to several protein kinases including the cdc2 and the MAP kinase families. Based on this homology, it is predicted to be missing approximately 130 amino acids from the amino terminus which is obtained via a second round of library screening with the amino terminal region of the obtained cDNA clone.

The other end of the cDNA contains the poly A sequence corresponding to
10 the 3' terminus of the mRNA from which it was obtained (Figure 14, Seq. I.D. No. 8).

Accordingly, based on initial cDNA (Figure 13), oligonucleotides (5'-
CCTCGGAGAATTTGGTAGATAAGG-3' (Seq. I.D. No. 9) and 5'-
AACATTGTGAAATGTCAGAAGCTTACAGATGACCAT-3' (Seq. I.D. No. 10))
15 were designed from the 5' end of the sense strand, and used to screen for cDNAs encoding the amino terminus of CSBP. The oligonucleotides were labelled at their 5' ends with polynucleotide kinase and γ -³²P-ATP. 10⁶ plaques from a GM-CSF stimulated human monocyte library constructed in λ ZAP were screened on duplicate
20 nitrocellulose filters which had been prewashed prior to hybridization in 2xSSPE, 0.1% SDS at 50°C. After blocking for 48h with 50% formamide, 6 x SSPE, 5 x Denhardt's and 100 μ g/ml sheared, denatured salmon sperm DNA, filters were hybridized in the same buffer with the above labelled oligonucleotides for 24 hours at 42°C. The filters were then washed twice with 2 x SSPE, 0.1% SDS at room
25 temperature, followed by two washes in 1 x SSPE, 0.1% SDS at 42°C and two washes in 0.5 x SSPE, 0.1% SDS at 42°C before detection of hybridizing plaques by autoradiography. Positive plaques which appeared on duplicate filters were picked and replated and the procedure repeated twice until unique plaques could be isolated and phagemid DNA released according to manufacturer's protocol
(Stratagene Cloning Systems, LaJolla, CA). The cDNAs were sequenced on an
30 Applied Biosystems automated DNA sequencer (ABI 373A) using universal and specific oligonucleotide primers and Taq polymerase cycle sequencing, and the sequences merged and examined using Lasergene software on a Macintosh IICI. Both strands were completely sequenced at least once in each cDNA clone.

Description of cDNAs.

A summary of the cDNAs isolated is illustrated schematically in Figure 15. There are four different cDNAs which have been completely sequenced and are identical in regions of overlap, with one exception to be described below. BP01/02 is the cDNA first isolated above, the partial sequence of which is given in Figures 13 and 14. The longest cDNA is 3.8 kb long (N5) Seq. I.D. No. 11 and contains 370 nucleotides of 5' untranslated sequence, a 1.1kb coding region and 2.4kb of 3' untranslated sequence. The extreme 3' end is terminated by a poly A stretch characteristic of mRNA, and is preceded by the expected consensus sequence for polyadenylation. The N7 cDNA has a 3' untranslated region of only 1.4kb terminating in a site and poly A run suggesting an alternative polyadenylation site. On a Northern blot a probe derived from the coding region hybridizes to an ca. 4.2kb mRNA suggesting that the longest cDNA isolated is close to full length.

The coding translates into a protein of 360 amino acids with calculated molecular weight of 41.5 kDa, matching the size of the protein identified by photoaffinity crosslinking with ¹²⁵I-labelled Compound IV (Figure 16). The predicted isoelectric point (ca. 5.6) is also close to that observed (Ca. 5.0). Examination of the sequence indicates that it contains both the tryptic peptide sequence ITAAQ... and the cyanogen bromide sequence LNNIVK...(boxed) obtained by sequencing of the CSAIDs binding protein in THP.1 cells. These sequences are preceded by the appropriate cleavage sites (arrows). The predicted size of the cyanogen bromide fragment (8kDa) matches the size of the fragment which remains associated with the ¹²⁵I-labelled radiophotoaffinity label [Compound IV] after cyanogen bromide treatment of the CSAIDs binding protein.

The N13 cDNA (Figure 15) Seq. I.D. No. 12 is identical to the other three cDNAs with the exception of a 75 nucleotide region starting at position 1054 of the N5 cDNA. This difference results in a protein of identical size with amino acids 230-255 altered. (Figure 17). The two different sequences are 43% identical at the nucleotide level, and 44% identical at the amino acid level. Without wishing to be bound by any particular theory, it is likely that the two variants result from alternative internal exon splicing, although allelic variation cannot be excluded. For ease of description, two proteins are referred to herein as CSBP1 (corresponding to the N5 cDNA) and CSBP2 (corresponding to the N13 cDNA).

Comparison of the CSBP sequence to proteins in the GenBank/EMBL or Swissprot databases indicated close homology to a family of proteins known as MAP (Mitogen Activated Protein) or erk (extracellular regulated) kinases (Boulton, et al., "Erks; A Family of Protein Serine-Threonine Kinases that are Activated and Tyrosine Phosphorylated in Response to Insulin and NGF", *Cell*, **65**: 663-675 (1993). This family of protein kinases is conserved from yeast to man as indicated in the phylogenetic tree in Figure 18 with the closest published homologue being the yeast HOG1 gene (Brewster et al., *Science* **259**: 1760-63 (1993). An alignment of the CSBPs with selected members of this family (Figure 19) shows a conservation of all 11 protein kinase motifs (I through XI), including residues identical in all protein kinases (bold) (Hanks et al., *Science*, **241**: 42-52 (1988). Two boxed motifs in regions VI and VIII indicate that the kinases phosphorylate serines and threonines (Hanks et al., 1988). Hence the CSBPs are protein kinases.

A threonine and tyrosine in a TxY sequence (asterisks) proximal to domain VIII are known to be regulatory phosphorylation sites for Erk 1 and Erk 2 (Payne, et al., *EMBO J.*, **10**: 885-892, 1991). These two residues are phosphorylated by MEK (MAPK or ERK Kinase) in response to various extracellular signals, resulting in an activation of the serine/threonine kinase activity of the MAP kinases (Kosako, et al., *EMBO J.*, **12**: 787-794 (1993). The conservation of these amino acids in the CSBPs suggests that they are also regulated by a MEK in response to extracellular stimuli such as LPS. These findings suggest that the CSBPs lie within a cascade of protein phosphorylation events which communicate cell surface stimuli to events such as translational regulation, within the cell. Much of the behavior of the CSBP in suitably stimulated cells can be predicted based on analogy with the known properties and behavior of the MAP kinases (Marshall, et al., *Curr. Opin. Genetics & Develop.*, **4**: 82-89 (1994).

A multiple tissue Northern blot with a coding region cDNA probe suggests expression of CSBP mRNA in most tissues. A Southern blot at high stringency (0.1% SSPE, 0.1% SDS) suggested a single gene; however lower stringency washes may reveal closely related kinases. Gene mapping experiments using a panel of human/mouse hybrid cell lines available commercially indicated that the gene for CSBP resides on human chromosome 6.

Expression in *E. coli*

To confirm that the proteins encoded by the isolated cDNAs can bind to CSAIDs, the cDNAs were expressed in *E. coli* and yeast. In *E. coli* the CSBPs were expressed as fusion proteins with β -galactosidase and/or an enterokinase cleavable
5 FLAG epitope tag (Figure 20) (FLAG is a commercial epitope for which reagents are available through IBI-Kodak). In the latter case this was achieved by the design of a synthetic oligonucleotide linker with an initiation site, antibody recognition
10 sequence, and enterokinase cleavage site. Proteins were expressed under the control of either the pLac (eg Bluescript KS vector from Stratagene, LaJolla, CA.) or λ pL (Shatman, et al., N.Y. Acad. Sci., 478: 233-248 (1986)) promoters and the radiophotoaffinity probe [Compound IV] shown to specifically crosslink proteins of the expected sizes in cell lysates. Lysates also contain Compound IA specific
15 binding activity. One can conclude that both CSBP1 and CSBP2 are the molecular targets of the CSAIDs within cells.

Protein expressed in *E. coli* was purified by passage over an affinity matrix containing a monoclonal antibody to the FLAG epitope according to manufacturer's instructions.

Expression in Yeast

20 An alternative system for expression of CSBP is *Saccharomyces cerevisiae*, not only for purification but also to assess function. The yeast *HOG1* (High Osmolarity Glycerol response) gene, (Brewster et al., surpa) encodes a MAP kinase which is a close homologue of CSBP. Mutant *hog1D* strains show reduced growth on high-osmolarity medium and functional complementation of this phenotype with
25 CSBP was tested.

CSBP2 was engineered for yeast expression as follows. A *Xho*I site was introduced at the initiation codon of CSBP2 by the polymerase chain reaction (Mullis, and Faloona, Method in Enzymol., 155: 335-50 (1987) using the following oligonucleotide primers: 5'-cgccctcgagatgtctcaggagagggccacg-3' Seq. I.D. No. 13
30 and 3'-ctaagacctaaaacctgaccg-5', Seq. No. 14. The 525-bp PCR fragment was digested with *Xho*I and *Bgl*II and subcloned into the same sites in p138NBU, a modification of p138NB (McHale et al., Mol. Pharm., 39: 109-113 (1991) in which the TRP1 selectable marker was replaced with URA 3. The resulting plasmid was then digested with *Bgl*II and *Sal*I and ligated with a *Bgl*II *Xho*II fragment
35 containing the 3' end of CSBP2. The final construct contains partial 2micron sequences for maintenance at high copy number, with CSBP2 mRNA expression

driven by the copper-inducible CUP1 promoter and terminated by the yeast CYC1 transcriptional terminator. Plasmid p138NBU-CSBPN13B was found encode the wild-type CSBP2 protein. Transformations of parent (YPH499 MATa ura3-52 lys2-801^{am} ade2-101 rrp1-D63 his3D200 leu2-D1) and hog1D (JBY10 [YPH499 + hog1::TRP1]) strains (Brewster, et al., J. Bacteriol. 153: 163-168 (1983) Ura⁺ prototrophs were isolated and grown to A₅₄₀ of 1.0 in synthetic complete medium lacking uracil (Hicks et al., Genetics 83: 245 (1976). CSBP2 expression was induced by the addition of 150 mM CuSO₄. Cells were harvested at 5 hr, resuspended 20 mM Tris-HCl pH7, 1 mM MgCl₂, 1 mM

10 phenylmethylsulfonylfluoride and disrupted by vortexing in the presence of 0.45 mm glass beads. Extracts were centrifuged at 1,500 x g for 5 min at 4°.

Radiophotoaffinity probe (Compound IV) was shown to specifically crosslink a protein of the expected size in lysates of both p138NBU-CSBPN13A and p138NBU-CSBPN13B, which was not present in wild type or hog1D strains

15 containing control plasmid (p138NBU) and grown under similar conditions. Lysates also contained ³H Compound Ia specific binding activity, Therefore both CSB1 and CSB2 bind CSAIDS.

The proteins of this invention are preferably made by recombinant genetic engineering techniques. The isolated nucleic acids particularly the DNAs can be

20 introduced into expression vectors by operatively linking the DNA to the necessary expression control regions (e.g. regulatory regions) required for gene expression. The vectors can be introduced into the appropriate host cells such as prokaryotic (e.g., bacterial), or eukaryotic (e.g., yeast or mammalian) cells by methods well known in the art (Ausubel et al., supra). The coding sequences for the desired proteins

25 having been prepared or isolated, can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative

30 bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), a baculovirus insect cell system, , YCp19 (Saccharomyces). See, generally, "DNA Cloning": Vols. I & II, Glover et al., eds. IRL Press Oxford (1985) (1987) and; T. Maniatis et al. "Molecular Cloning", Cold Spring Harbor Laboratory

35 (1982).

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this
5 expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The subunit antigens of the present invention can be expressed using, for example, the *E. coli* *lac* promoter or the protein A gene (*spa*) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739;
10 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be
15 turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and
20 orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in
25 some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression
30 vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. Alternatively, gene fusions may be created whereby the gene encoding the
35 binding protein of interest is fused to a gene encoding a product with other desirable properties. For example, a fusion partner could provide known assayable

activity (e.g. enzymatic) which could be used as an alternative means of selecting the binding protein. The fusion partner could be a structural element, such as a cell surface element such that the binding protein (a normally cytosolic component) could be displayed on the cell surface in the form of a fusion protein. It may also be desirable to produce mutants or analogs of the protein of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis and the formation of fusion proteins, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of prokaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,578,355; 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491. pSV2neo (as described in J. Mol. Appl. Genet. 1:327-341) which uses the SV40 late promoter to drive expression in mammalian cells or pCDNA1neo, a vector derived from pCDNA1 (Mol. Cell Biol. 7:4125-29) which uses the CMV promoter to drive expression. Both these latter two vectors can be employed for transient or stable (e.g. using G418 or hygromycin resistance) expression in mammalian cells. Insect cell expression systems, e.g., Drosophila, are also useful, see for example, PCT applications US 89/05155 and US 91/06838 as well as EP application 88/304093.3 and Baculovirus expression systems.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates or recovered from the cell membrane fraction. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform E. coli and pooling

and screening individual colonies using polyclonal serum or monoclonal antibodies to the desired binding protein.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides is not particularly preferred.

The binding proteins of the present invention or their fragments comprising at least one epitope can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with a binding protein of the present invention, or its fragment, or a mutated binding protein. Serum from the immunized animal is collected and treated according to known procedures. When serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography or other known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B-lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies and T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the protein of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Alternatively, genes encoding the monoclonals of interest may be isolated from the hybridomas by PCR techniques known in the art and cloned and expressed in the appropriate vectors. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual proteins against which they are directed. The antibodies of this invention, whether polyclonal or monoclonal have additional utility in that they may be employed reagents in immunoassays, RIA, ELISA, and the like. In addition they can be used to isolate the CSBP from human cells and determine the effect of different stimuli and compounds on the phosphorylation state and protein kinase activity of endogenous CSBP. The antibodies could be used to establish a tissue culture based assay for

discovery or modification of novel compounds which block the phosphorylation or kinase activity of CSBP. An example of such an assay would be to incubate human monocytes or monocytic cell lines with a compound or compound mixture prior to treatment with LPS for a defined time period, followed by immunoprecipitation of CSBP with antibody and assessment of its phosphorylation state via immunoblot or chromatography or measurement of its kinase activity with appropriate protein or peptide substrate.

This invention provides a method for determining whether a ligand previously not known to bind to a CSBP can bind to such a protein. The method comprises contacting the ligand to be identified with cytosolic fraction from THP.1 cells and measuring its ability to compete with a known radioactive CSAID, as described above, in a CSAIDs binding assay. Alternative methods include contacting the ligand to be identified with a whole-cell expressing the coding sequence of a CSBP under conditions sufficient for binding of ligands previously identified as binding to such a receptor. In other embodiments cell membrane fractions comprising the CSBP fusions or isolated CSBP free or immobilized on solid supports may be used to measure binding of the ligand to be tested. When recombinant cells are used for purposes of expression of the CSBP it is preferred to use cells with little or no endogenous CSBP activity so that binding if any is due to the presence of the expressed protein of interest. As mentioned previously, a specifically designed indicator of receptor binding can be constructed. For example a fusion protein can be made by fusing the CSBP of this invention with a protein domain which is sensitive to CSBP/ ligand binding. Such a domain referred to here as an indicator domain is capable, itself, or in association with accessory molecules, of generating an analytically detectable signal which is indicative of receptor ligand binding. A variation of this approach is to express CSBP as a fusion protein (e.g., fused to FLAG peptide) in THP.1 or other mammalian cells, and to use the fusion peptide as a means of isolating the recombinant CSBP after suitable stimulation and pretreatment of THP.1 cells. Such expression can be achieved with numerous mammalian expression vectors which utilize viral promoters, eg CMV, RSV and polyadenylation sequences, et. SV40, bovine growth hormone, and a selectable marker such as G418 or hygromycin for selection of stable transfectants.

Cytosolic preparations from transfected or transformed cells expressing such fusions may be employed. All of the above techniques that are useful for ligand identification are also useful in drug screening and drug development protocols.

Alternatively, the purified recombinant protein could be used to substitute for crude THP.1 cell lysates in a competitive binding assay with Compound Ia. This assay is useful to screen for novel compound which bind CSBP, or as a way to assess alterations to compound which is known to bind. The availability of purified protein
5 allows alternative configurations of the assay from those described previously for the crude material. For example, if the protein is covalently linked to a tag, such a protein binding site for configuration in a colorimetric assay, e.g., conjugated antibody, or to an enzyme for direct detection of enzyme activity, e.g., horseradish peroxidase or alkaline phosphatase, binding to novel compounds displayed on a solid matrix
10 could be detected. Such compounds could include low molecular weight organic molecules, peptides, peptoids, and proteins. In the latter case, the protein can be used as a way to isolate other proteins in its signaling cascade, for example, those that are in the pathway for activation of cytokine translation in activated monocytes. The protein may also be used to isolate naturally occurring regulatory molecules within
15 mammalian cells that act by a CSAIDs binding mechanism. Finally, the protein can be used to identify target peptides displayed on the surface of phage.

The knowledge that the CSBPs encode protein kinases suggest that recombinant forms can be used to establish a protein kinase activity. Typically this would involve the direct incubation of CSBP with a protein or peptide substrate in the
20 presence of γ -³²P-ATP, followed by the measurement of radioactivity incorporated into the substrate by separation and counting. Separation methods include immunoprecipitation, conjugation of substrate to a bead allowing separation by centrifugation or determination of incorporation by scintillation proximity assay, SDS-PAGE followed by autoradiography or biosensor analysis. While the specific
25 substrates are not yet known, candidates include CSBP itself (autophosphorylation) and peptides related to known MAP kinase substrates. Other substances might be discovered by incubating CSBP with random peptides conjugated to solid supports or displayed by phage (see above) or by incubation of CSBP with mammalian cell lysates (e.g. THP.1 cell lysates) and γ -³²P-ATP, followed by separation of the labelled
30 target proteins, and sequencing. Kinase activity may also be detected by use of antiphosphotyrosine antibodies. The protein kinase activity of CSBP may require incubation with a specific MEK. This may be achieved by preincubating CSBP with lysates from stimulated eukaryotic cells (e.g., LPS treated THP.1 cells) and ATP. Alternatively, it may be possible to isolate a more active form of CSBP from HOG1
35 deletion strains of yeast expressing the human CSBP and grown in high osmolarity conditions.

These assays permit the discovery and modification of compounds which inhibit CSBP kinase activity in vitro. Such compounds would be expected to block cytokine synthesis in a comparable fashion to the compounds described herein. They could also lead to the discovery of novel substrates which themselves may be viable targets for discovery of novel compounds which block cytokine production.

It is expected that CSBPs, like other MAP kinases, will be activated by a MEK, hence the recombinant protein would allow the establishment of a second assay which measures the ability of CSBP to be phosphorylated by putative MEKs. In this case fractions from stimulated cell lysates (eg THP.1 cells stimulated with LPS) are incubated with CSBP in the presence of γ - ^{32}P -ATP, and the incorporation of ^{32}P -label into CSBP measured by separation and counting. Separation can be achieved in a number of ways: one way is to use a CSBP fused to an peptide or protein and separate via affinity chromatography or immunoprecipitation with the peptide or protein directed antibody. Alternatively the CSBP can be directly conjugated to beads or bound through a fusion peptide or protein (e.g., FLAG (peptide), glutathionine-S-transferase) and separated by centrifugation after incubation with cell lysates. Also tyrosine phosphorylation of CSBP could be detected by immunoprecipitation or immunoblot with commercially available anti-phosphotyrosine antibodies.

These assays can be used to discover compounds which block the activation of CSBP protein kinase activity and to improve the potency of already discovered compounds. These compounds would be expected to have utility due to their blocking of cytokine synthesis. The assays are also useful to discover novel MEKs which themselves may become targets for novel compounds which would block cytokine synthesis.

The ability of human CSBP to rescue a HOG1 deletion strain upon growth in conditions of high osmolarity allows for the direct screening of compounds which block CSBP activity in vivo. For example, compounds could be screened for their ability to block growth of a CSBP +/HOG1- yeast strain in high osmolarity but which have no effect on growth of the same strain in standard osmolarity or on a CSBP-/HOG1+ in high osmolarity. The sensitivity of the yeast based assay can be increased by introducing host mutations that affect the cell membrane and permeability (Gaber, et al., Mol. Cell. Biol. 9: 3447-3456. (1989).

In a compound screening embodiment of this invention, the CSBP in isolated, immobilized or cell bound form is contacted with a plurality of candidate molecules and those candidates are selected which bind to and interact with the protein. The

binding or interaction can be measured directly by using radioactively labeled candidate of interest or indirectly by measuring an effect resulting from the interaction or binding of the candidate compound. Alternatively, the candidate compounds can be subjected to a competition screening assays, in which a known ligand, preferably
5 labeled with an analytically detectable reagent, most notably radioactivity, is introduced with the compounds to be tested and the compound's capacity to inhibit or enhance the binding of the labeled ligand is measured. Compounds are screened for their increased affinity and selectivity for the CSBP.

To illustrate this aspect of the invention a natural product screen was
10 performed.

The standard assay in which bound ligand is separated from free by exclusion chromatography using mini-columns was used to initiate a screening effort. Approximately 625 marine extracts, 202 microbial extracts and 233 extracts of plant material were tested for inhibition of ^3H -Compound I binding to THP.1 cytosol.
15 Two extracts were confirmed as antagonists of this binding, with IC_{50} 's of around 200 and 80 $\mu\text{g/ml}$ respectively. This low hit-rate (0.2%) coupled with the failure to observe inhibition by any of a selected group of "nuisance extracts" indicates that the assay is sufficiently selective and robust to support a screening effort. While the potency of these two hits is rather weak, they were nevertheless accepted as leads for
20 isolation of their active principle so that the primary assay could be evaluated as well as identification of the bioactive compounds.

The two extracts were subsequently fractionated and characterized.

Further refinement of the binding assay to facilitate high throughout screening can be achieved by the minor modification of separating bound ligand from free ligand
25 using spin columns.

This invention also contemplates pharmaceutical compositions comprising compounds when identified by the above methods and a pharmaceutically acceptable carrier. Pharmaceutical compositions of proteinaceous drugs of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly
30 or intravenously. The compositions for parenteral administration will commonly comprise a solution of the compounds of the invention or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These
35 solutions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as

required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the compound of the invention in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected
5 primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 50 mg of a compound of the invention. Similarly, a pharmaceutical composition of the invention
10 for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of a compound of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

15 The compounds described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional proteins and art-known lyophilization and reconstitution techniques can be employed.

In situations where the identified drug is non-proteineous, it may be
20 administered alone or in combination with pharmaceutically acceptable carriers. The proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice. For example, they may be administered orally in the form of tablets or capsules containing such excipients as starch, milk sugar, certain types of clay and so forth. They may be
25 administered sublingually in the form of troches or lozenges in which the active ingredient is mixed with sugar and corn syrups, flavoring agents and dyes; and then dehydrated sufficiently to make it suitable for pressing into a solid form. They may be administered orally in the form of solutions which may be injected parenterally, that is, intramuscularly, intravenously or subcutaneously. For parenteral
30 administration they may be used in the form of a sterile solution containing other solutes, for example, enough saline or glucose to make the solution isotonic.

The physician will determine the dosage of the present therapeutic agents which will be most suitable and it will vary with the form of administration and the particular compound chosen, and furthermore, it will vary with the particular patient
35 under treatment. He will generally wish to initiate treatment with small dosages substantially less than the optimum dose of the compound and increase the dosage by

small increments until the optimum effect under the circumstances is reached. It will generally be found that when the composition is administered orally, larger quantities of the active agent will be required to produce the same effect as a smaller quantity given parenterally. The compounds are useful in the same manner as other
5 serotonergic agents and the dosage level is of the same order of magnitude as is generally employed with these other therapeutic agents. The therapeutic dosage will generally be from 1 to 10 milligrams per day and higher although it may be administered in several different dosage units. Tablets containing from 0.5 to 10 mg. of active agent are particularly useful.

10 Depending on the patient condition, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease in an amount sufficient to cure or at least partially arrest the disease and its complications. In prophylactic applications, compositions containing the
15 present compounds or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance.

Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention should provide a quantity
20 of the compounds of the invention sufficient to effectively treat the patient.

The nucleic acid embodiment of this invention is particularly useful in providing probes capable of specific hybridization with human CSBP sequences. Probing technology is well known in the art and it is appreciated that the size of the probes can vary widely but it is preferred that the probe be at least 15 nucleotides in
25 length. It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. This invention contemplates, for example using receptor encoding probes in the diagnostic
30 evaluation of disease states characterized by an abnormal, i.e. increased or decreased level of receptor gene expression. Alternatively, the probes can be used to identify individuals carrying chromosomal or molecular mutations in the gene encoding the receptor. Depending on the conditions employed by the ordinary skilled artisan, the probes can be used to identify and recover additional examples of this receptor (in its
35 genomic or cDNA form) from other cell types and individuals. As a general rule the

more stringent the hybridization conditions the more closely related genes will be that are recovered.

Also within the scope of this invention are antisense oligonucleotides predicated upon the sequences disclosed herein for the CSBP. Synthetic
5 oligonucleotides or related antisense chemical structural analogs are designed to recognize and specifically bind to a target nucleic acid encoding the receptor gene and inhibit gene expression, e.g., the translation of the gene when the target nucleic acid is mRNA. Although not wishing to be bound to a particular theory for the mechanism of action of antisense drugs, it is believed that such drugs can act by one
10 or more of the following mechanisms: by binding to mRNA and inducing degradation by endogenous nucleases such as RNase I or by inhibiting the translation of mRNA by inhibiting its binding to regulatory factors or ribosomal components necessary for productive protein synthesis. Additionally the antisense sequences can be use as components of a complex macromolecular arrays in which the sequences are
15 combined with ribozyme sequences or reactive chemical groups and are used to specifically target mRNAs of interest and degrade or chemically modify said mRNAs. The general field of antisense technology is illustrated by the following disclosures which are incorporated herein by reference for purposes of background (Cohen, J.S., Trends in Pharm. Sci. 10:435(1989) and Weintraub, H.M. Scientific American
20 Jan.(1990) at page 40).

This invention also contemplates the use of the DNA sequences disclosed herein in gene therapy. Because CSBP is a protein kinase it is possible to make a site specific mutant which is inactive as a kinase but will block activation of the
endogenous CSBP when coexpressed in the same cell, i.e., it is a dominant negative
25 mutant (Kolch et al., Nature 349: 426-428 (1991). The DNA encoding this mutant protein could be used in gene therapy to reduce chronic inflammation. There are many vector and delivery systems available to direct DNA into target cells in vivo, e.g. adenovirus, retroviruses.

This invention also contemplates antibodies, monoclonal or polyclonal
30 directed to epitopes corresponding to amino acid sequences disclosed herein from the CSBP. Particularly important regions of the receptor for immunological purposes are those regions associated with ligand binding domains of the protein. Antibodies directed to the regions are particularly useful in diagnostic and therapeutic applications because of their effect upon protein- ligand interaction. Methods for the
35 production of polyclonal and monoclonal antibodies are well known, see for example Chap. 11 of Ausubel et al. (supra).

This invention also provides pharmaceutical compositions comprising an effective amount of antibody or fragment thereof directed against the CSBP to block binding of the naturally occurring ligands to that protein in order to treat or ameliorate disease states associated with protein activation.

5 Transgenic, non-human, animals may be obtained by transfecting appropriate fertilized eggs or embryos of a host with nucleic acids encoding the CSBP disclosed herein, see for example U.S. Patents 4,736,866; 5,175,385; 5,175,384 and 5,175,386. The resultant transgenic animal may be used as a model for the study of CSBP/ligand interaction. Particularly, useful transgenic animals are those which display a
10 detectable phenotype associated with the expression of the protein. Drugs may then be screened for their ability to reverse or exacerbate the relevant phenotype. This invention also contemplates operatively linking the CSBP coding gene to regulatory elements which are differentially responsive to various temperature or metabolic conditions, thereby effectively turning on or off the phenotypic expression in
15 response to those conditions.

The nucleic acid probes disclosed herein can be used to clone the cognate version of the human CSBP gene from a desired experimental animal species; for example the murine version. Strains of mice can be developed in which said gene has been eliminated by conventional gene knock-out technology. The gene can then be
20 substituted/or replaced by the human CSBP DNA of this invention to yield a mouse for screening candidate drugs in vivo. Similar gene knockout and human protein inhibition studies can also be performed with yeast.

The purified protein of this invention is also useful in a reagent for structural studies with and without bound drug candidates as a means for the rational design of
25 novel drugs affecting CSBP. For example, the recombinant protein may be used to derive the structure of the protein alone or complexed with Compound Ia and related compounds through X-ray crystallography, NMR or modelling from published structures of related protein kinases, e.g., CSK. A structure fosters an understanding of how the inhibitory compounds bind, and can lead to the design or discovery of
30 further compounds which can block CSBP activity and hence be inhibitors of cytokine synthesis. There are now several examples of such structure-based design for other protein targets, e.g., HIV protease. Given the similarity of CSBP to several other kinases (e.g. the MAP and CDC kinases), such structural information will be useful in designing novel compounds which inhibit other members of the kinase
35 family.

SEQUENCE LISTING

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 (B) COMPUTER: IBM PC compatible
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 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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 (B) FILING DATE:
 (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- 40 (A) APPLICATION NUMBER: US 08/123,175
 (B) FILING DATE: 17-SEP-1993

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5

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10

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

20

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (G) CELL TYPE: Monocyte
- (H) CELL LINE: THP.1

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

35

Ile Thr Ala Ala Gln Ala Leu Ala His Ala Tyr Phe Ala Gln Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE
20 (H) CELL LINE: THP.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25 Xaa Gln Leu Leu Asn Asn Ile Val Lys Phe Gln Lys Leu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

40

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(G) CELL TYPE: MONOCYTE

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCYCAYGCTA YTTYGCYCAR TA

22

10 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20 (iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

25 (A) ORGANISM: HOMO SAPIENS

(G) CELL TYPE: MONOCYTE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

30

AAYAAYATYK TBAARTTYCA AA

22

(2) INFORMATION FOR SEQ ID NO:5:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

10 (G) CELL TYPE: MONOCYTE

(H) CELL LINE: THP.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15 Asn Ile Val Lys Cys Gln Lys Leu Thr

1 5

(2) INFORMATION FOR SEQ ID NO:6:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 285 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

30 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

35 (G) CELL TYPE: MONOCYTE

(H) CELL LINE: THP.1

(ix) FEATURE:

(A) NAME/KEY: CDS

40 (B) LOCATION: 1..285

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAC ATT GTG AAA TGT CAG AAG CTT ACA GAT GAC CAT GTT CAG TTC CTT 48
 5 Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu
 1 5 10 15
 ATC TAC CAA ATT CTC CGA GGT CTA AAG TAT ATA CAT TCA GCT GAC ATA 96
 10 Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile
 20 25 30
 ATT CAC AGG GAC CTA AAA CCT AGT AAT CTA GCT GTG AAT GAA GAC TGT 144
 15 Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys
 35 40 45
 GAG CTG AAG ATT CTG GAT TTT GGA CTG GCT CGG CAC ACA GAT GAT GAA 192
 Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu
 50 55 60
 20 ATG ACA GGC TAC GTG GCC ACT AGG TGG TAC AGG GCT CCT GAG ATC ATG 240
 Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met
 65 70 75 80
 CTG AAC TGG ATG CAT TAC AAC CAG ACA GGT GGT ATT TGG GTC AAG 285
 25 Leu Asn Trp Met His Tyr Asn Gln Thr Gly Gly Ile Trp Val Lys
 85 90 95

(2) INFORMATION FOR SEQ ID NO:7:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 95 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

40 Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu
 1 5 10 15

Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile
 20 25 30
 5 Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys
 35 40 45
 Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu
 50 55 60
 10 Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met
 65 70 75 80
 Leu Asn Trp Met His Tyr Asn Gln Thr Gly Gly Ile Trp Val Lys
 15 85 90 95

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 392 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

 25 (ii) MOLECULE TYPE: cDNA

 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO
 30 (v) ORIGINAL SOURCE:
 (A) ORGANISM: HOMO SAPIENS
 (G) CELL TYPE: MONOCYTE
 (H) CELL LINE: THP.1
 35 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 1..392

40

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAAGTCCCAA TCCTCCCAA CCACAGCAAG TTGAATTTAT CAACCATGTT GGGTTGTAAA 60
 5 TGCTCGTGTG ATTTCTTACA AGAAATACCT GCTCTGAATA TTTTGTAAAT AAAGGTCTTT 120
 GCACATGTGA CCCACAATAC GTGTTAGGAG CCTGCATGCT CTGGAAGCCT GGACTCTAAG 180
 CTGGAGCTCT TGGAAGAGCT CTTGGGTTTC TGAGCATAAT GCTCCCATCT CCTGATTTCT 240
 10 CTGAACAGAA AACAAAAGAG AGAATGAGGG AAATTGCTAT TTTATTTGTA TTGATGAACT 300
 TGGCTGTAAT CAGTTATGCC GTATAGGATG TCAGACAATA CCACTGGTTA AAATAAGCC 360
 15 TATTTTTTCAA ATTTAAAAAA AAAAAAAAAA AA 392

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 25 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 30 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: Monocyte

35

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTCGGAGAA TTTGGTAGAT AAGG

24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(G) CELL TYPE: Monocyte

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AACATTGTGA AATGTCAGAA GCTTACAGAT GACCAT

36

(2) INFORMATION FOR SEQ ID NO:11:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3813 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

30

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

35

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

40

(G) CELL TYPE: Monocyte

(ix) **FEATURE:**

(A) NAME/KEY: CDS

(B) LOCATION: 379..1461

5

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

	CCTCCTGGTA TAATCTGGAA CCGCGACCAC TGGAGCCTTA GCGGGCGCAG CAGCTGGAAC	60
10	GGGAGTACTG CGACGCAGCC CGGAGTCGGC CTTGTAGGGG CGAAGGTGCA GGGAGATCGC	120
	GGCGGGCGCA GTCTTGAGCG CCGGAGCGCG TCCCTGCCCT TAGCGGGGCT TGCCCCAGTC	180
	GCAGGGGCAC ATCCAGCCGC TCGGGCTGAC AGCAGCCGCG CGCGCGGGAG TCTGCGGGGT	240
15	CGCGGCAGCC GCACCTGCGC GGGCGACCAG CGCAAGGTCC CCGCCCGGCT GGGCGGGCAG	300
	CAAGGGCCGG GGAGAGGGTG CGGGTGCAGG CGGGGGCCCC ACAGGGCCAC CTTCTTGCCC	360
20	GGCGGCTGCC GCTGGAAA ATG TCT CAG GAG AGG CCC ACG TTC TAC CGG CAG	411
	Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln	
	1 5 10	
	GAG CTG AAC AAG ACA ATC TGG GAG GTG CCC GAG CGT TAC CAG AAC CTG	459
25	Glu Leu Asn Lys Thr Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu	
	15 20 25	
	TCT CCA GTG GGC TCT GGC GCC TAT GGC TCT GTG TGT GCT GCT TTT GAC	507
30	Ser Pro Val Gly Ser Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp	
	30 35 40	
	ACA AAA ACG GGG TTA CGT GTG GCA GTG AAG AAG CTC TCC AGA CCA TTT	555
	Thr Lys Thr Gly Leu Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe	
	45 50 55	
35	CAG TCC ATC ATT CAT GCG AAA AGA ACC TAC AGA GAA CTG CGG TTA CTT	603
	Gln Ser Ile Ile His Ala Lys Arg Thr Tyr Arg Glu Leu Arg Leu Leu	
	60 65 70 75	

	AAA CAT ATG AAA CAT GAA AAT GTG ATT GGT CTG TTG GAC GTT TTT ACA	651
	Lys His Met Lys His Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr	
	80 85 90	
5	CCT GCA AGG TCT CTG GAG GAA TTC AAT GAT GTG TAT CTG GTG ACC CAT	699
	Pro Ala Arg Ser Leu Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His	
	95 100 105	
10	CTC ATG GGG GCA GAT CTG AAC AAC ATT GTG AAA TGT CAG AAG CTT ACA	747
	Leu Met Gly Ala Asp Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr	
	110 115 120	
15	GAT GAC CAT GTT CAG TTC CTT ATC TAC CAA ATT CTC CGA GGT CTA AAG	795
	Asp Asp His Val Gln Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys	
	125 130 135	
20	TAT ATA CAT TCA GCT GAC ATA ATT CAC AGG GAC CTA AAA CCT AGT AAT	843
	Tyr Ile His Ser Ala Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn	
	140 145 150 155	
	CTA GCT GTG AAT GAA GAC TGT GAG CTG AAG ATT CTG GAT TTT GGA CTG	891
	Leu Ala Val Asn Glu Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu	
	160 165 170	
25	GCT CGG CAC ACA GAT GAT GAA ATG ACA GGC TAC GTG GCC ACT AGG TGG	939
	Ala Arg His Thr Asp Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp	
	175 180 185	
30	TAC AGG GCT CCT GAG ATC ATG CTG AAC TGG ATG CAT TAC AAC CAG ACA	987
	Tyr Arg Ala Pro Glu Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr	
	190 195 200	
35	GTT GAT ATT TGG TCA GTG GGA TGC ATA ATG GCC GAG CTG TTG ACT GGA	1035
	Val Asp Ile Trp Ser Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly	
	205 210 215	
40	AGA ACA TTG TTT CCT GGT ACA GAC CAT ATT AAC CAG CTT CAG CAG ATT	1083
	Arg Thr Leu Phe Pro Gly Thr Asp His Ile Asn Gln Leu Gln Gln Ile	
	220 225 230 235	

	ATG CGT CTG ACA GGA ACA CCC CCC GCT TAT CTC ATT AAC AGG ATG CCA	1131
	Met Arg Leu Thr Gly Thr Pro Pro Ala Tyr Leu Ile Asn Arg Met Pro	
	240 245 250	
5	AGC CAT GAG GCA AGA AAC TAT ATT CAG TCT TTG ACT CAG ATG CCG AAG	1179
	Ser His Glu Ala Arg Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys	
	255 260 265	
10	ATG AAC TTT GCG AAT GTA TTT ATT GGT GCC AAT CCC CTG GCT GTC GAC	1227
	Met Asn Phe Ala Asn Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp	
	270 275 280	
15	TTG CTG GAG AAG ATG CTT GTA TTG GAC TCA GAT AAG AGA ATT ACA GCG	1275
	Leu Leu Glu Lys Met Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala	
	285 290 295	
20	GCC CAA GCC CTT GCA CAT GCC TAC TTT GCT CAG TAC CAC GAT CCT GAT	1323
	Ala Gln Ala Leu Ala His Ala Tyr Phe Ala Gln Tyr His Asp Pro Asp	
	300 305 310 315	
	GAT GAA CCA GTG GCC GAT CCT TAT GAT CAG TCC TTT GAA AGC AGG GAC	1371
	Asp Glu Pro Val Ala Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp	
	320 325 330	
25	CTC CTT ATA GAT GAG TGG AAA AGC CTG ACC TAT GAT GAA GTC ATC AGC	1419
	Leu Leu Ile Asp Glu Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser	
	335 340 345	
30	TTT GTG CCA CCA CCC CTT GAC CAA GAA GAG ATG GAG TCC TGAGCACCTG	1468
	Phe Val Pro Pro Pro Leu Asp Gln Glu Glu Met Glu Ser	
	350 355 360	
35	GTTTCTGTTC TGTTGATCCC ACTTCACTGT GAGGGGAAGG CCTTTTCACG GGAACCTCC	1528
	AAATATTATT CAAGTGCCTC TTGTTGCAGA GATTTCCTCC ATGGTGGAAG GGGGTGTGCG	1588
	TGCGTGTGCG TGCGTGTTAG TGTGTGTGCA TGTGTGTGTC TGTCTTTGTG GGAGGGTAAG	1648
40	ACAATATGAA CAACTATGA TCACAGTGAC TTTACAGGAG GTTGTGGATG CTCCAGGGCA	1708
	GCCTCCACCT TGCTCTTCTT TCTGAGAGTT GGCTCAGGCA GACAAGAGCT GCTGTCCTTT	1768

TAGGAATATG TTCAATGCAA AGTAAAAAAA TATGAATTGT CCCCATCCC GGTCATGCTT 1828
 TTGCCACTTT GGCTTCTCCT GTGACCCAC CTTGACGGTG GGGCGTAGAC TTGACAACAT 1888
 5 CCCACAGTGG CACGGAGAGA AGGCCCATAC CTTCTGGTTG CTTGAGACCT GACACCGTCC 1948
 CTCAGTGATA CGTACAGCCA AAAAGGACCA ACTGGCTTCT GTGCACTAGC CTGTGATTAA 2008
 10 CTTGCTTAGT ATGTTTCTCA GATCTTGACA GTATATTGA AACTGTAAAT ATGTTTGTGC 2068
 CTTAAAAGGA GAGAAGAAAG TGTAGATAGT TAAAAGACTG CAGCTGCTGA AGTTCTGAGC 2128
 CGGGCAAGTC GAGAGGGCTG TTGGACAGCT GCTTGTGGGC CCGGAGTAAT CAGGCAGCCT 2188
 15 TCATAGGCGG TCATGTGTGC ATGTGAGCAC ATGCGTATAT GTGCGTCTCT CTTTCTCCCT 2248
 CACCCCCAGG TGTTGCCATT TCTCTGCTTA CCCTTCACCT TTGGTGCAGA GGTTCCTTGA 2308
 20 ATATCTGCCC CAGTAGTCAG AAGCAGGTTT TTGATGTCAT GTACTTCCTG TGTACTCTTT 2368
 ATTTCTAGCA GAGTGAGGAT GTGTTTTGCA CGTCTTGCTA TTTGAGCATG CACAGCTGCT 2428
 TGTCTGCTC TCTTCAGGAG GCCCTGGTGT CAGGCAGGTT TGCCAGTGAA GACTTCTTGG 2488
 25 GTAGTTTAGA TCCCATGTCA CCTCAGCTGA TATTATGGCA AGTGATATCA CCTCTCTTCA 2548
 GCCCCTAGTG CTATTCTGTG TTGAACACAA TTGATACTTC AGGTGCTTTT GATGTGAAAA 2608
 30 TCATGAAAAG AGGAACAGGT GGATGTATAG CATTTTTATT CATGCCATCT GTTTTCAACC 2668
 AACTATTTTT GAGGAATTAT CATGGGAAAA GACCAGGGCT TTTCCAGGA ATATCCCAA 2728
 CTTGGAAC AAGTTATTCT CTTCACTCCC AATAACTAAT GCTAAGAAAT GCTGAAATC 2788
 35 AAAGTAAAA ATTAAGCCC ATAAGGCCAG AACTCCTTT TGCTGTCTTT CTCTAAATAT 2848
 GATTACTTTA AAATAAAAA GTAACAAGGT GTCTTTTCCA CTCCTATGGA AAAGGTCTT 2908
 40 CTTGGCAGCT TAACATTGAC TTCTTGGTTT GGGGAGAAAT AAATTTTGT TCAGAATTTT 2968

	GTATATTGTA GGAATCCCTT TGAGAATGTG ATTCCTTTTG ATGGGGAGAA AGGGCAAATT	3028
	ATTTTAATAT TTTGTATTTT CAACTTTATA AAGATAAAAT ATCCTCAGGG GTGGAGAAGT	3088
5	GTCGTTTTCA TAACTTGCTG AATTTTCAGGC ATTTTGTCTT ACATGAGGAC TCATATATTT	3148
	AAGCCTTTTG TGTAATAAGA AAGTATAAAG TCACTTCCAG TGTGGGCTGT GTGACAGAAT	3208
	CTGTATTTTG GGCCAAGGTG TTTCCATTTC TCAATCAGTG CAGTGATACA TGTACTCCAG	3268
10	AGGGACGGGT GGACCCCTG AGTCAACTGG AGCAAGAAGG AAGGAGGCAG ACTGATGGCG	3328
	ATCCCTCTC ACCCGGGACT CTCCCCCTT CAAGGAAAGT GAACCTTTAA AGTAAAGGCC	3388
15	TCATCTCCTT TATTGCAGTT CAAATCCTCA CCATCCACAG CAAGATGAAT TTTATCAGCC	3448
	ATGTTTGGTT GTAAATGCTC GTGTGATTTC CTACAGAAAT ACTGCTCTGA ATATTTTGTA	3508
	ATAAAGGTCT TTGCACATGT GACCACATAC GTGTTAGGAG GCTGCATGCT CTGGAAGCCT	3568
20	GGACTCTAAG CTGGAGCTCT TGGAAGAGCT CTTCGGTTTC TGAGCATAAT GCTCCCATCT	3628
	CCTGATTCT CTGAACAGAA AACAAAAGAG AGAATGAGGG AAATTGCTAT TTTATTTGTA	3688
25	TTCATGAAGT TGGCTGTAAT CAGTTATGCC GTATAGGATG TCAGACAATA CCACTGGTTA	3748
	AAATAAGGCC TATTTTTCAA ATTTAAAAA AAAAAAAAAA AAGTCCAGCA ATTCGTTAC	3808
	TTATG	3813
30		

(2) INFORMATION FOR SEQ ID NO:12:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 360 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr
1 5 10 15

15 Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser
20 25 30

Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu
35 40 45

20
Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His
50 55 60

	Ala	Lys	Arg	Thr	Tyr	Arg	Glu	Leu	Arg	Leu	Leu	Lys	His	Met	Lys	His
25	65					70					75					80

Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu
85 90 95

30 Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp
 100 105 110

Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln
115 120 125

35 Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala
130 135 140

Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu
40 145 150 155 160

	Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp	
	165	170 175
5	Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu	
	180	185 190
	Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser	
	195	200 205
10	Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro	
	210	215 220
	Gly Thr Asp His Ile Asn Gln Leu Gln Gln Ile Met Arg Leu Thr Gly	
	225	230 235 240
15	Thr Pro Pro Ala Tyr Leu Ile Asn Arg Met Pro Ser His Glu Ala Arg	
	245	250 255
	Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn	
20	260	265 270
	Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met	
	275	280 285
25	Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala	
	290	295 300
	His Ala Tyr Phe Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala	
	305	310 315 320
30	Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu	
	325	330 335
	Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro	
35	340	345 350
	Leu Asp Gln Glu Glu Met Glu Ser	
	355	360

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1423 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: Monocyte

(ix) FEATURE:

20

- (A) NAME/KEY: CDS
 (B) LOCATION: 227..1309

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

25

CCACTCCTGG TATAATCTCG CCCAGTCGC AGGGGCACAT CCAGCCGCTG CGGCTGACAG 60

CAGCCGCGCG CGCGGGAGTC TGCGGGGTCG CGGCAGCCGC ACCTGCGCGG GCGACCAGCG 120

30 CAAGGTCCCC GCCCGCTGG GCGGGCAGCA AGGGCCGGGG AGAGGGTGCG GGTGCAGGCG 180

GGGGCCCCAC AGGGCCACCT TCTTGCCCGG CGGCTGCCGC TGGAAA ATG TCT CAG 235
 Met Ser Gln

1

35

GAG AGG CCC ACG TTC TAC CGG CAG GAG CTG AAC AAG ACA ATC TGG GAG 283
 Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr Ile Trp Glu

5

10

15

	GTG CCC GAG CGT TAC CAG AAC CTG TCT CCA GTG GGC TCT GGC GCC TAT	331
	Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser Gly Ala Tyr	
	20 25 30 35	
5	GGC TCT GTG TGT GCT GCT TTT GAC ACA AAA ACG GGG TTA CGT GTG GCA	379
	Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu Arg Val Ala	
	40 45 50	
10	GTG AAG AAG CTC TCC AGA CCA TTT CAG TCC ATC ATT CAT GCG AAA AGA	427
	Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His Ala Lys Arg	
	55 60 65	
15	ACC TAC AGA GAA CTG CGG TTA CTT AAA CAT ATG AAA CAT GAA AAT GTG	475
	Thr Tyr Arg Glu Leu Arg Leu Leu Lys His Met Lys His Glu Asn Val	
	70 75 80	
20	ATT GGT CTG TTG GAC GTT TTT ACA CCT GCA AGG TCT CTG GAG GAA TTC	523
	Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu Glu Glu Phe	
	85 90 95	
25	AAT GAT GTG TAT CTG GTG ACC CAT CTC ATG GGG GCA GAT CTG AAC AAC	571
	Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp Leu Asn Asn	
	100 105 110 115	
30	ATT GTG AAA TGT CAG AAG CTT ACA GAT GAC CAT GTT CAG TTC CTT ATC	619
	Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu Ile	
	120 125 130	
35	TAC CAA ATT CTC CGA GGT CTA AAG TAT ATA CAT TCA GCT GAC ATA ATT	667
	Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile Ile	
	135 140 145	
40	CAC AGG GAC CTA AAA CCT AGT AAT CTA GCT GTG AAT GAA GAC TGT GAG	715
	His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys Glu	
	150 155 160	
45	CTG AAG ATT CTG GAT TTT GGA CTG GCT CGG CAC ACA GAT GAT GAA ATG	763
	Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu Met	
	165 170 175	

	ACA GGC TAC GTG GCC ACT AGG TGG TAC AGG GCT CCT GAG ATC ATG CTG	811
	Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met Leu	
	180 185 190 195	
5	AAC TGG ATG CAT TAC AAC CAG ACA GTT GAT ATT TGG TCA GTG GGA TGC	859
	Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser Val Gly Cys	
	200 205 210	
10	ATA ATG GCC GAG CTG TTG ACT GGA AGA ACA TTG TTT CCT GGT ACA GAC	907
	Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro Gly Thr Asp	
	215 220 225	
15	CAT ATT GAT CAG TTG AAG CTC ATT TTA AGA CTC GTT GGA ACC CCA GGG	955
	His Ile Asp Gln Leu Lys Leu Ile Leu Arg Leu Val Gly Thr Pro Gly	
	230 235 240	
20	GCT GAG CTT TTG AAG AAA ATC TCC TCA GAG TCT GCA AGA AAC TAT ATT	1003
	Ala Glu Leu Leu Lys Lys Ile Ser Ser Glu Ser Ala Arg Asn Tyr Ile	
	245 250 255	
25	CAG TCT TTG ACT CAG ATG CCG AAG ATG AAC TTT GCG AAT GTA TTT ATT	1051
	Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn Val Phe Ile	
	260 265 270 275	
30	GGT GCC AAT CCC CTG GCT GTC GAC TTG CTG GAG AAG ATG CTT GTA TTG	1099
	Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met Leu Val Leu	
	280 285 290	
35	GAC TCA GAT AAG AGA ATT ACA GCG GCC CAA GCC CTT GCA CAT GCC TAC	1147
	Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala His Ala Tyr	
	295 300 305	
40	TTT GCT CAG TAC CAC GAT CCT GAT GAT GAA CCA GTG GCC GAT CCT TAT	1195
	Phe Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala Asp Pro Tyr	
	310 315 320	
40	GAT CAG TCC TTT GAA AGC AGG GAC CTC CTT ATA GAT GAG TGG AAA AGC	1243
	Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu Trp Lys Ser	
	325 330 335	

CTG ACC TAT GAT GAA GTC ATC AGC TTT GTG CCA CCA CCC CTT GAC CAA 1291
 Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro Leu Asp Gln
 340 345 350 355

5 GAA GAG ATG GAG TCC TGAGCACCTG GTTCTGTTC TGTGATCCC ACTTCACTGT 1346
 Glu Glu Met Glu Ser
 360

GAGGGGAAGG CCTTTTCACG GGAAGTCTCC AATATTATT CAAGTGCCAA AAAGGTCCAG 1406

10 CAATTCGTT ACTTATG 1423

(2) INFORMATION FOR SEQ ID NO:14:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 360 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr
 1 5 10 15

Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser
 20 25 30

30 Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu
 35 40 45

Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His
 35 50 55 60

Ala Lys Arg Thr Tyr Arg Glu Leu Arg Leu Leu Lys His Met Lys His
 65 70 75 80

40 Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu
 85 90 95

Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp
 100 105 110

5 Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln
 115 120 125

Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala
 130 135 140

10 Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu
 145 150 155 160

Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp
 15 165 170 175

Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu
 180 185 190

20 Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser
 195 200 205

Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro
 210 215 220

25 Gly Thr Asp His Ile Asp Gln Leu Lys Leu Ile Leu Arg Leu Val Gly
 225 230 235 240

Thr Pro Gly Ala Glu Leu Leu Lys Lys Ile Ser Ser Glu Ser Ala Arg
 30 245 250 255

Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn
 260 265 270

35 Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met
 275 280 285

Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala
 290 295 300

40

His Ala Tyr Phe Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala
 305 310 315 320

Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu
 5 325 330 335

Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro
 340 345 350

10 Leu Asp Gln Glu Glu Met Glu Ser
 355 360

(2) INFORMATION FOR SEQ ID NO:15:

15

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

25

(iv) ANTI-SENSE: NO

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCCCTCGAG ATGTCTCAGG AGAGGCCAC G

31

(2) INFORMATION FOR SEQ ID NO:16:

35

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: CDNA

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10

GCCAGTCCAA AATCCAGAAT C

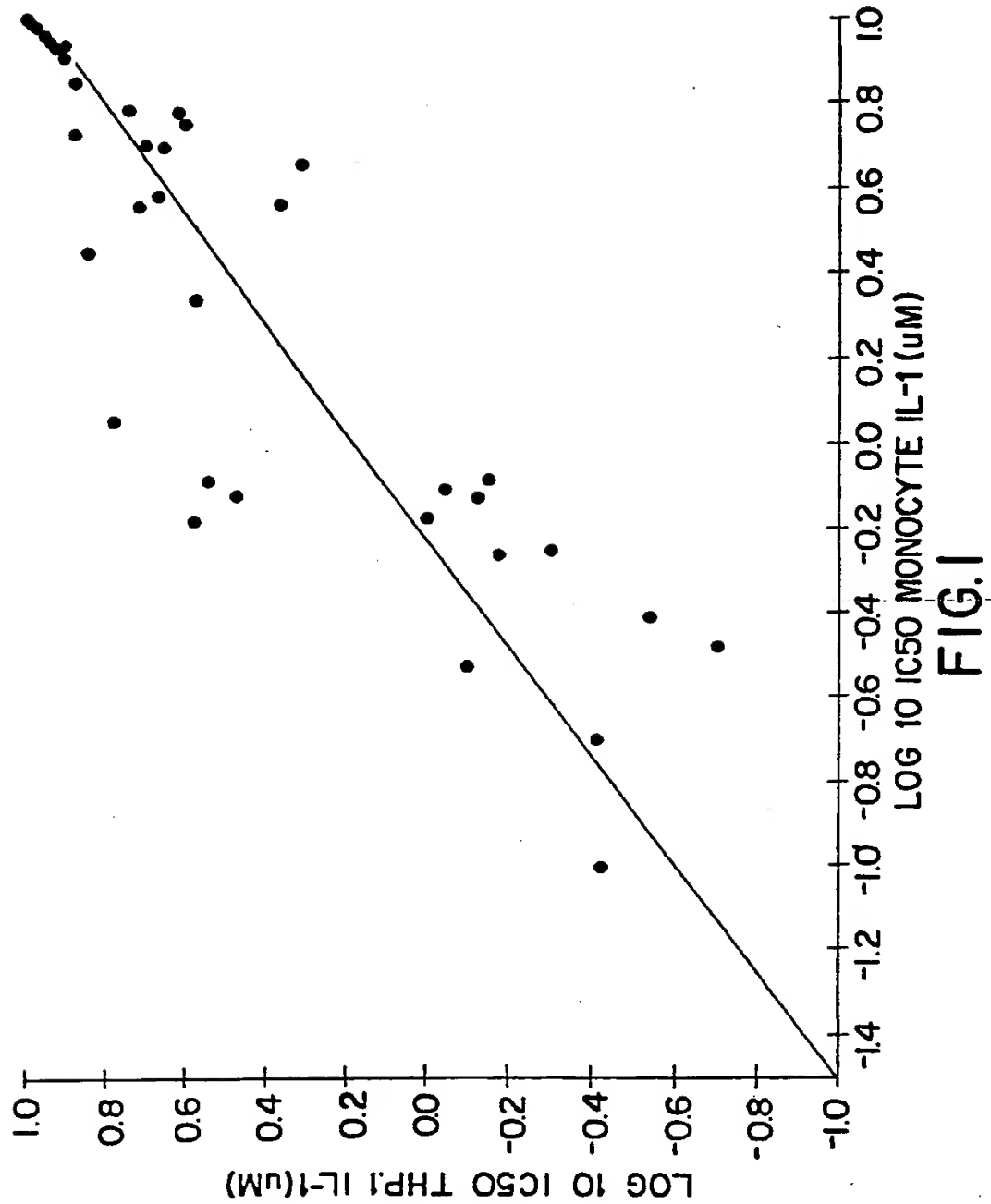
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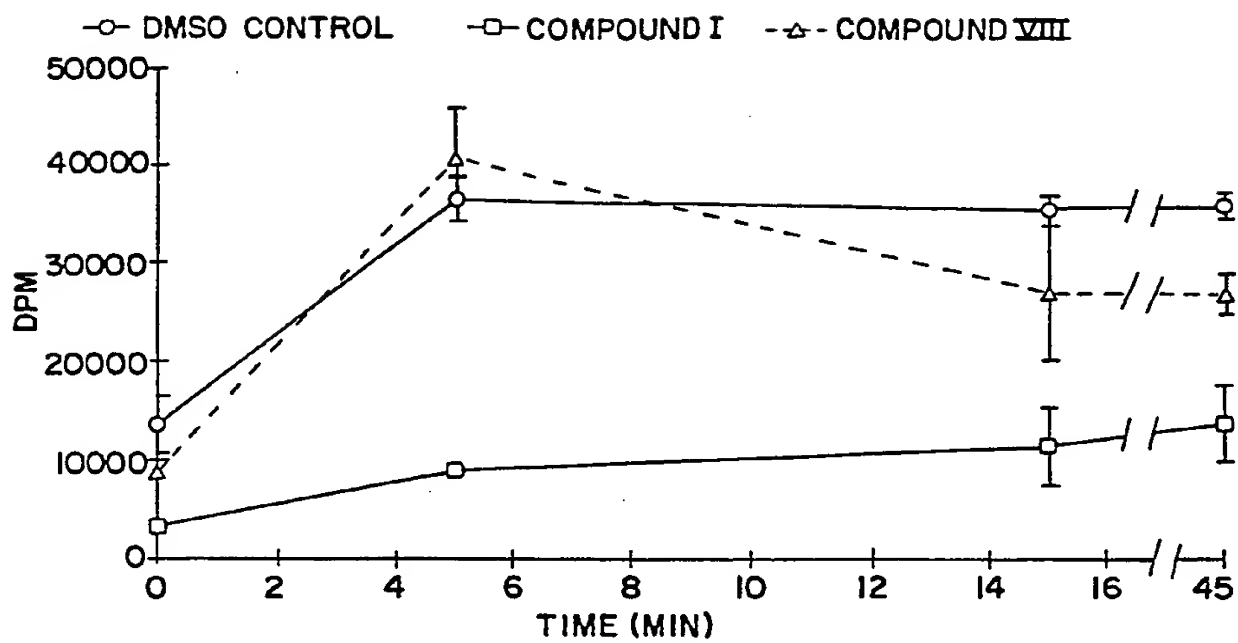
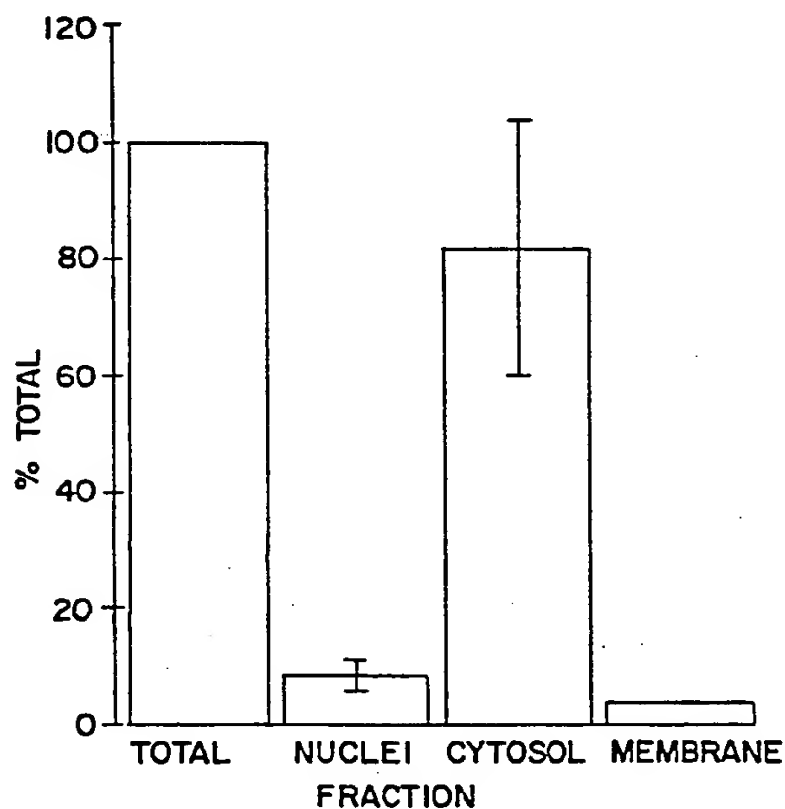
WHAT IS CLAIMED IS

1. An isolated nucleic acid molecule encoding a cytokine suppressive anti-inflammatory drug binding protein.
- 5 2. The molecule according to Claim 1 wherein said nucleic acid is DNA.
3. The molecule according to Claim 2 having the sequence characterized by Figures 21 and 22.
4. An isolated protein which is a human CSBP.
5. The protein according to Claim 4 characterized by the amino acid
10 sequence Figure 16.
6. The protein according to Claim 4 further characterized as being isolatable from human monocytes, having M_w of about 43,000 and having a pI of about 4.5.
7. A vector comprising the nucleic acid of Claim 1.
- 15 8. The vector according to Claim 7 which is a plasmid.
9. The plasmid according to Claim 8 which is a cloning plasmid.
10. The plasmid according to Claim 8 which is an expression plasmid.
11. A recombinant host cell comprising the vector of Claim 7.
12. The host cell according to Claim 11 which is a prokaryotic cell.
- 20 13. The host cell according to Claim 11 which is an eukaryotic cell.
14. A method for the production of CSBP comprising culturing a recombinant host cell capable of expressing CSBP in a medium and under conditions sufficient form such expression and recovering CSBP from the host cell.
15. A method for identifying a compound as a CSAID comprising: (a)
25 contacting a known CSAID labelled with an analytically detectable reagent with a CSBP under conditions sufficient to form a CSAID/CSBP complex; (b) contacting said complex with a sample comprising a compound to be identified; and (c) identifying the compound as a CSAID by detecting the ability of said compound to alter the amount of labelled CSAID in said complex.
- 30 16. The method according to Claim 15 wherein the CSBP is in a form selected from the group consisting of whole cells, cytosolic cell fractions, membrane cell fractions, and purified or partially purified form.
17. A method for identifying a compound as a CSAID comprising:
 - a. forming soluble cytosolic fraction from a cell expressing a
35 CSBP.

- 5 b. contact said fraction with a CSAID labelled with an
analytically detectable reagent under conditions sufficient to
form a reagent CSAID/CSBP complex;
- c. contacting said complex with a sample containing a CSAID;
and
- d. detecting the CSAID by measuring a decrease of the amount of
reagent in the labelled CSAID/CSBP complex.
18. The method according to Claim 17 wherein said cell is a human
monocyte.
- 10 20. The method according to Claim 17 wherein said cell is a recombinant
host cell.
21. The method according to Claim 17 wherein said reagent is a
radioactive label.
- 15 22. A method for identifying ligands capable of binding to a CSBP
comprising: contacting a recombinant host cell expressing a CSBP with a ligand to be
identified under conditions to permit binding and detecting the presence of any ligand-
bound protein.
23. The method according to Claim 21 wherein the recombinant host cell
expresses said CSBP at its cell surface.
- 20 24. The method according to Claim 21 wherein the protein or a membrane
fraction containing the protein is isolated from said cell prior to contacting with the
ligand to be identified.
25. An antagonist or agonist compound identified by the method of Claim
15.
- 25 26. A pharmaceutical composition comprising a compound identified by
the method of Claim 15 and a pharmaceutically acceptable carrier.
27. A antisense oligonucleotide having sequence capable of binding
specifically with any sequence of an mRNA molecule which encodes the human CSBP
containing the amino acid sequence of Figure 16 so as to prevent the translation
thereof.
- 30 28. An antibody directed to the human CSBP of Claim 5.
29. The antibody according to Claim 26 which is a monoclonal antibody.
30. A transgenic non-human mammal capable of expressing in any cell
thereof the DNA of Claim 3.

30. A method of screening compounds to identify those compounds which bind to a human CSBP comprising contacting the fusion protein comprising a CSBP domain and a binding protein/ligand binding indicator domain with a plurality of compound, under conditions to permit binding to the CSBP domain, and identifying those candidate drugs capable of enhancing or inhibiting the activity of the protein/ligand binding indicator domain.
- 5



**FIG.2****FIG.3**

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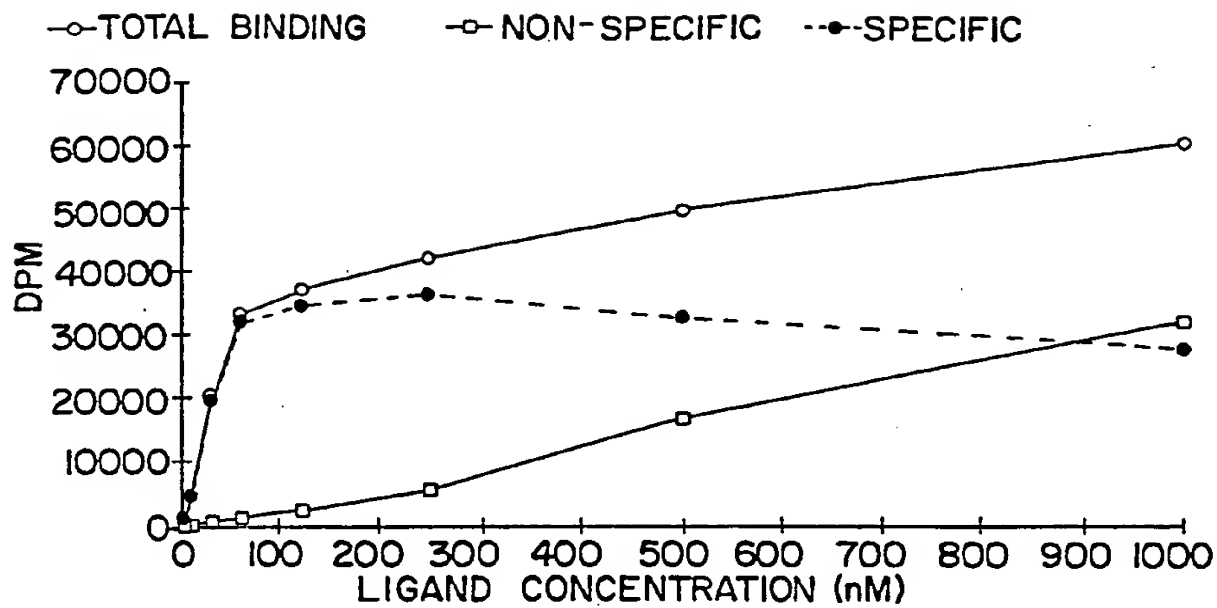


FIG.4A

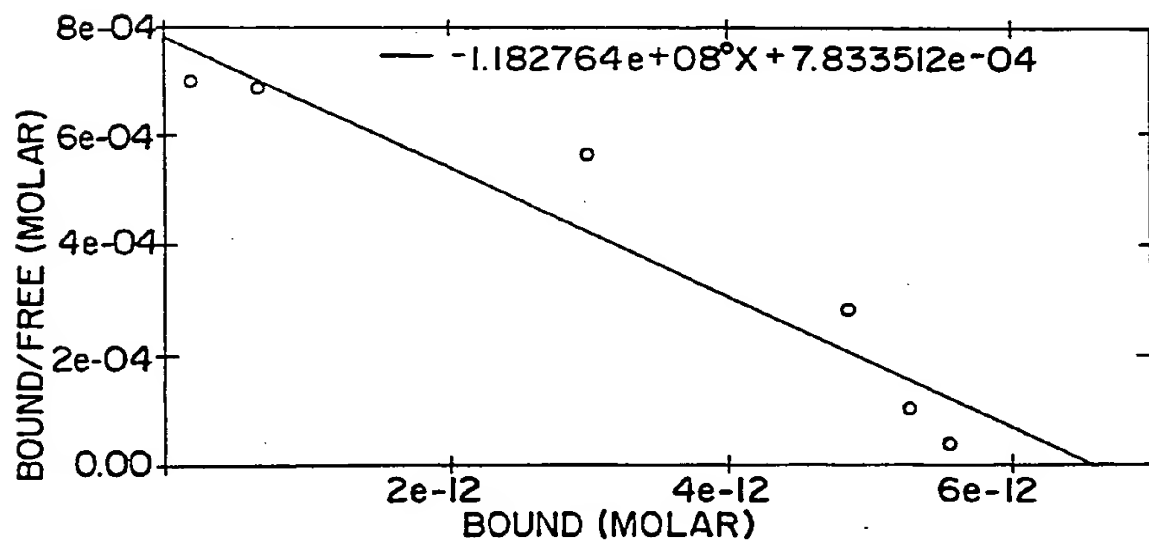


FIG.4B

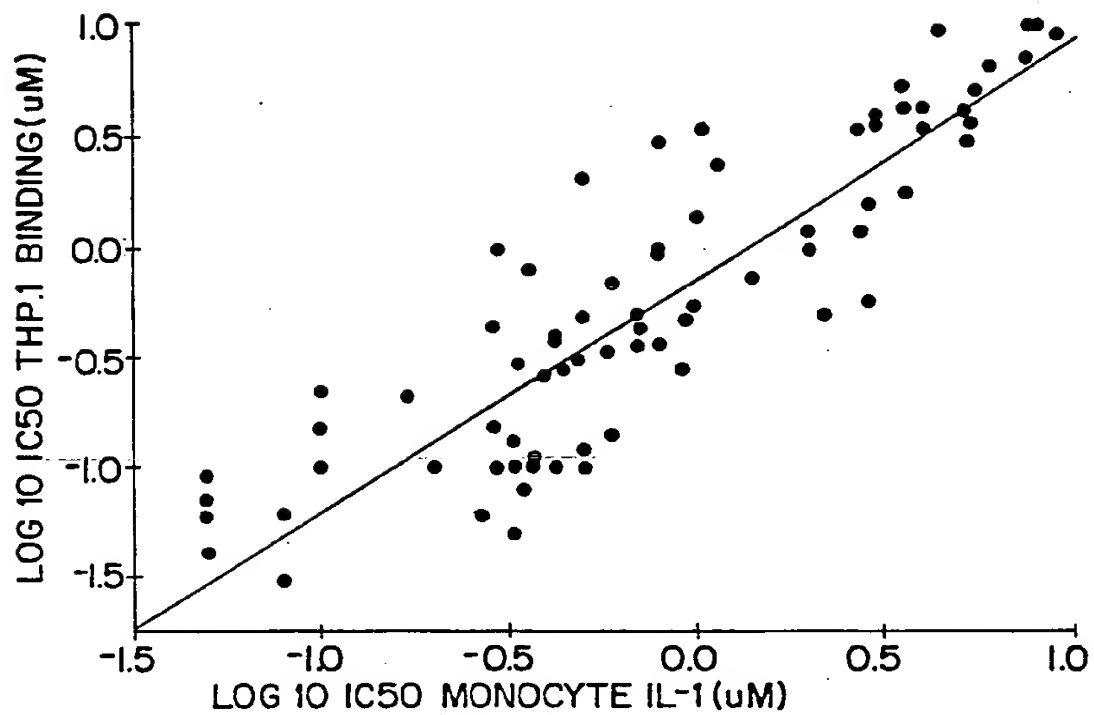
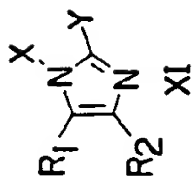


FIG.5



VALUES ARE IC₅₀, μM

COMPOUND	R1/R2	X-Y	IL-1	TNF	THP-1	CYTOSOL	BINDING
XI(a)	4-pyridyl/4-FPh	-CH ₂ CH ₂ S-	0.5	0.4			<0.1
XI(b)	4-FPh/4-pyridyl	-CH ₂ CH ₂ S-	>5	>5			10
XI(c)	4-pyridyl/4-FPh	-CH ₂ CH ₂ CH ₂ -	0.2	0.2			<0.1
XI(d)	4-FPh/4-pyridyl	-CH ₂ CH ₂ CH ₂ -	>5	>5			>10
XI(e)	4-pyridyl/4-MeSPh	-CH ₂ CH ₂ CH ₂ -	2.7	2.7			3.4
XI(f)	4-MeSPh/4-pyridyl	-CH ₂ CH ₂ CH ₂ -	>5	>5			5
XI(g)	4-pyridyl/4-FPh	-CH ₂ CH ₂ OAC,H	0.5	3			0.5
XI(h)	4-FPh/4-pyridyl	-CH ₂ CH ₂ OAC,H	5	5			>10

FIG. 6

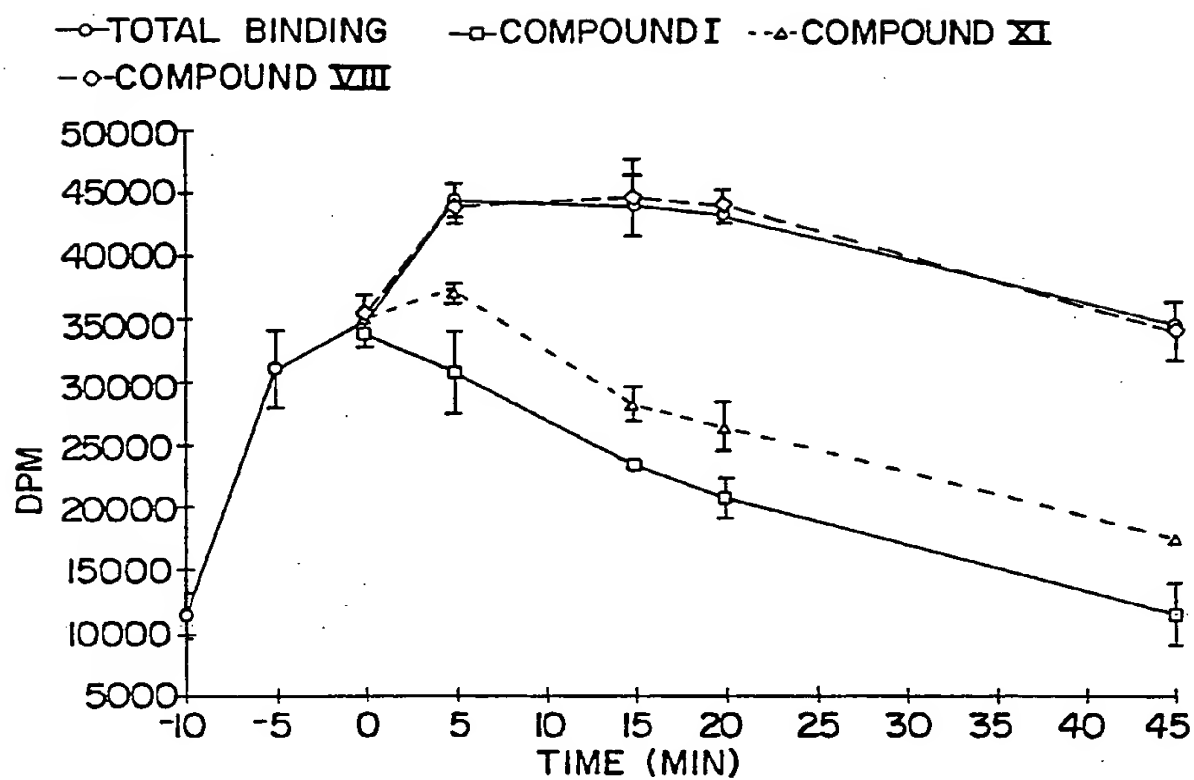


FIG.7

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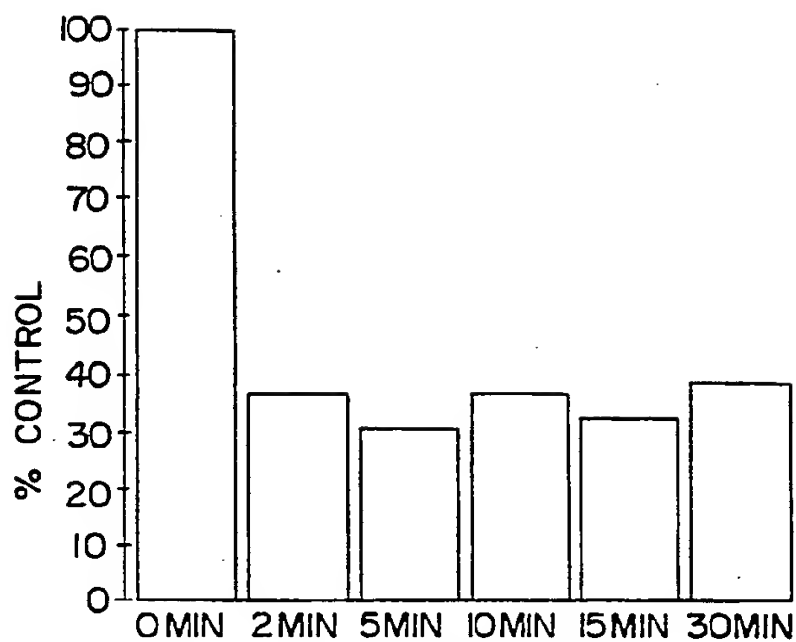
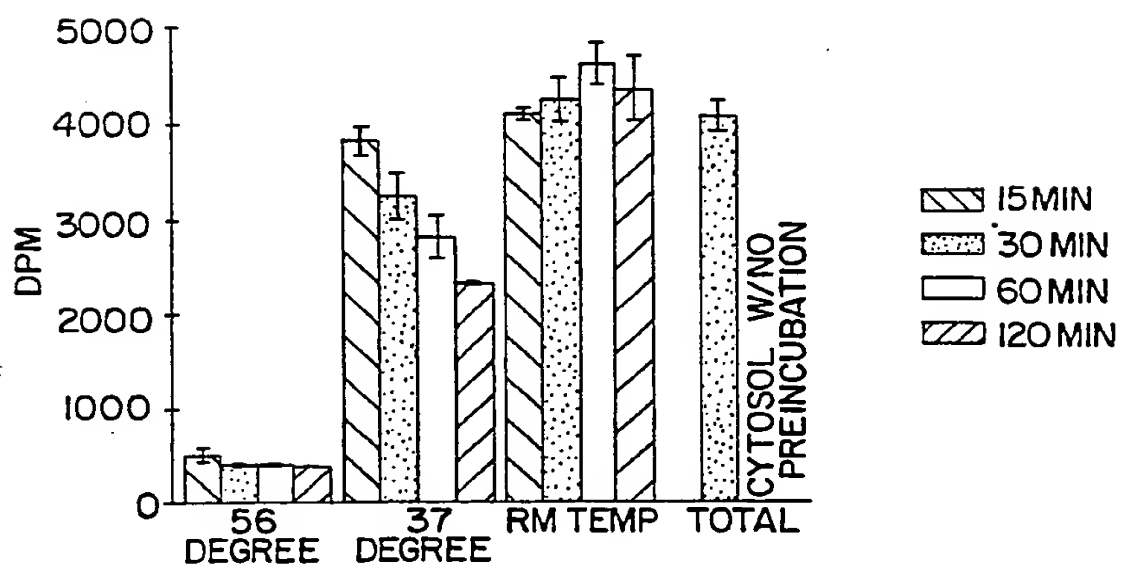


FIG.8A



TEMPERATURE

FIG.8B

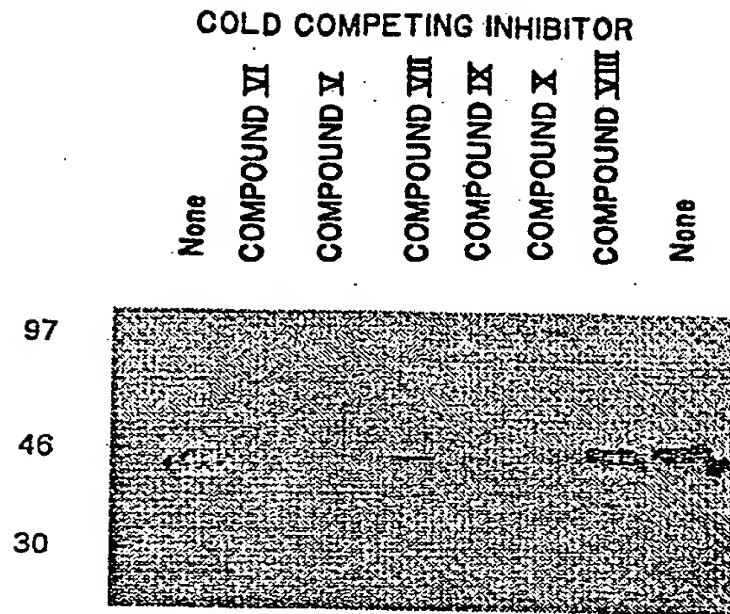


FIG.9

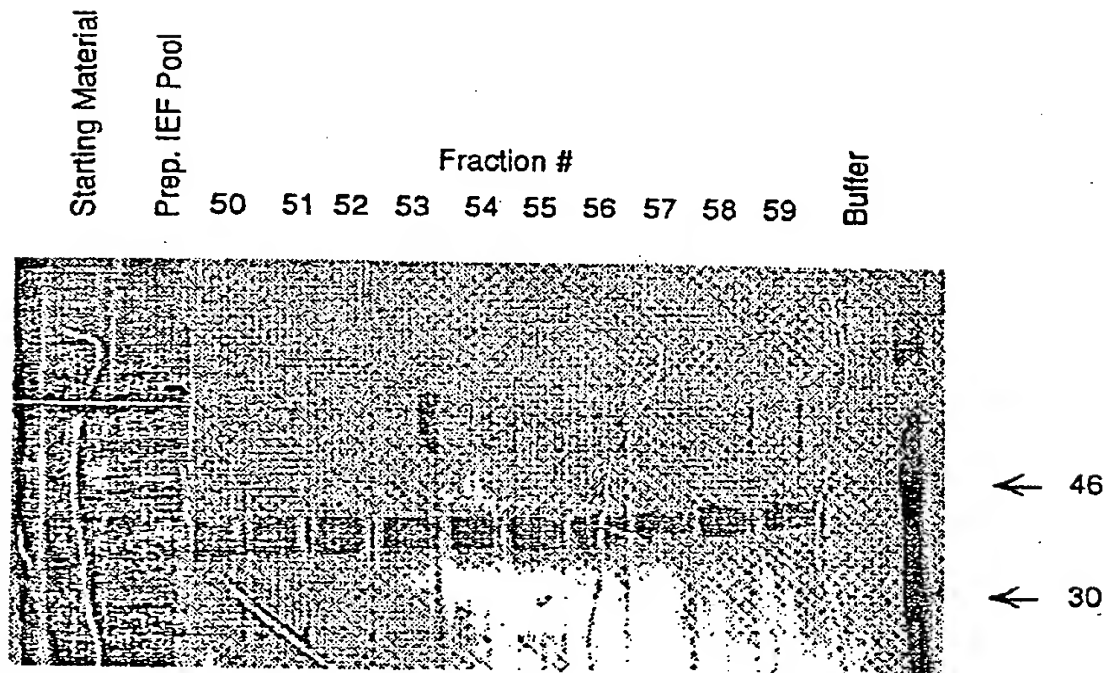


FIG.IIA

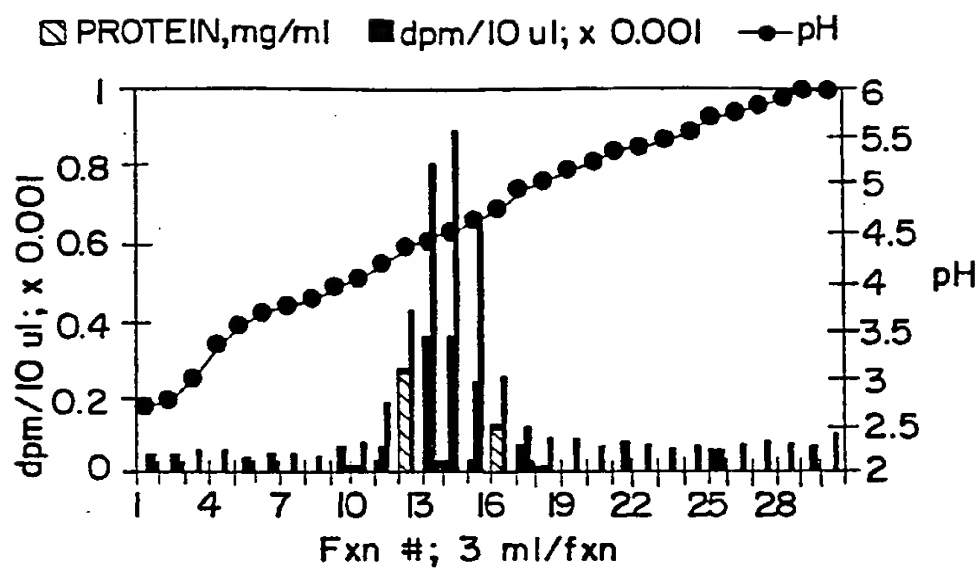


FIG.10

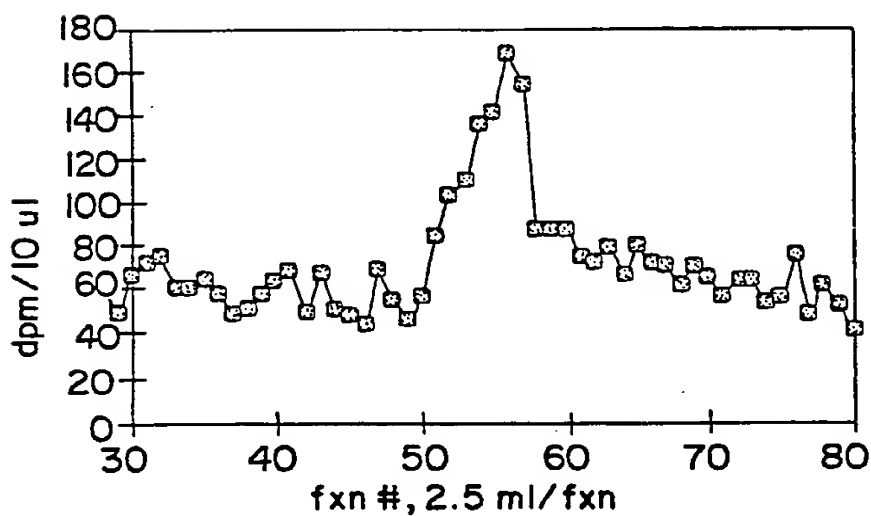


FIG.11B

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FIG.12

1 AACATTGTGAAATGTCAGAAAGCTTACAGATGACCATGTTTCAGTTCCTTATCTACCAAATT 60
AsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheLeuIleTyrGlnIle

61 CTCCGAGGTCTAAAGTATATACATTTCAGCTGACATAATTACAGGGACCTAAACCTAGT 120
LeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSer

121 AATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGATTTTGGACTGGCTCGGCAC 180
AsnLeuAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHis

181 ACAGATGATGAAATGACAGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATG 240
ThrAspAspGluMetThrGlyTyrValAlaThrArgTyrArgAlaProGluIleMet

241 CTGAACTGGATGCATTACAACACAGAGGTGGTATTGGGTCAAG 285
LeuAsnTrpMetHisTyrAsnGlnThrGlyGlyIleTrpValLys

FIG. 13

CAAGTCCCAATCCTCCCCAACCACAGCAAGTTGAATTTATCAACCATGTTGGGTTGTAA
TGCTCGTGTGATTTCTACAAGAAATACCTGCTCTGAATATTTTGTAAATAAGGTCTTT
GCACATGTGACCCACAATACGTGTTAGGAGCCTGCATGCTCTGGAAGCCTGGACTCTAAG
CTGGAGCTCTTGAAGAGCTCTTCGGTTTCTGAGCATAATGCTECCATCTCCTGATTTCT
CTGAACAGAAAACAAAAGAGAGAATGAGGGAAATTGCTATTTTATTTGTATTCATGAACT
TGGCTGTAATCAGTTATGCCGTATAGGATGTCAGACAATAACCACTGGTTAAAATAAGCC
TATTTTTCAAATTTAAAAA

FIG.14

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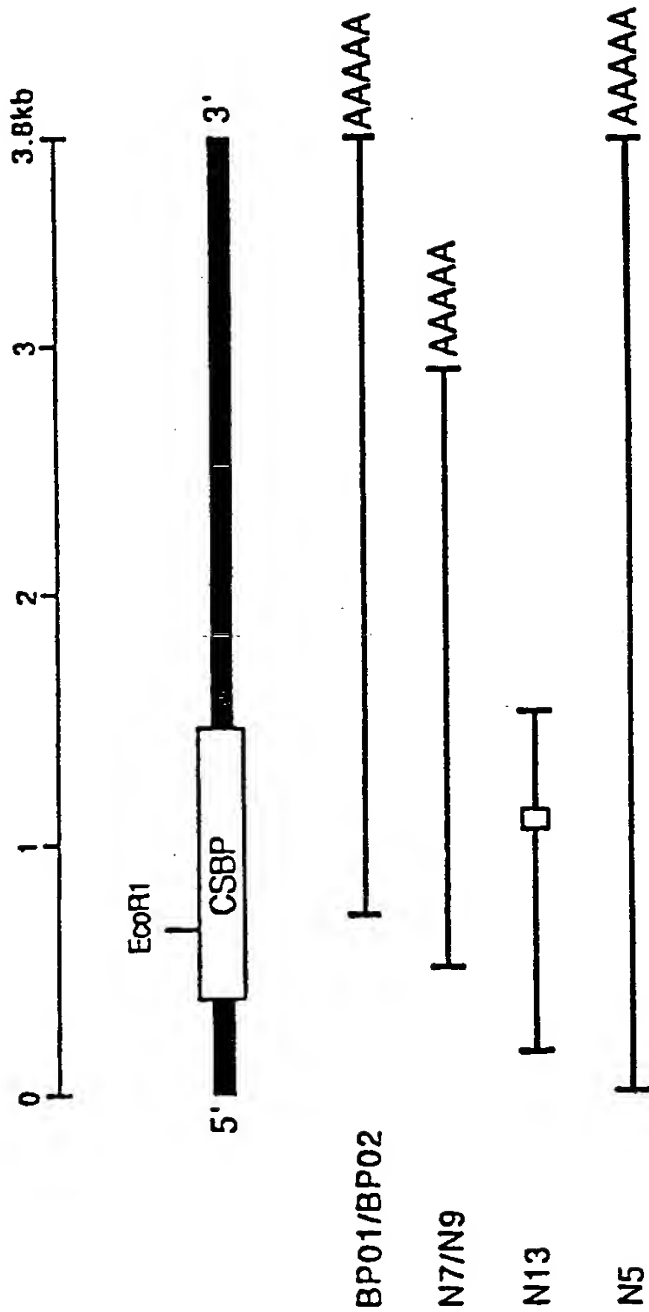


FIG.15

..355nts..GCCGCTGGAAAATGCTCTCAGGAGAGGCCACGTTCTACCGGCGAGGAGCTGAACAAGACAATCTGG 420
 M S Q E R P T F Y R Q E L N K T I W 18
 GAGGTGCCCGAGCGTTACCAGAACCTGTCTCCAGTGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGAC 495
 E V P E R Y Q N L S P V G S G A Y G S V C A A F D 43
 ACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTTCAGTCCCATCTTCATGCGAAAAGAACC 570
 T K T G L R V A V K K L S R P F Q S I I H A K R T 68
 TACAGAGAACTGCGGTTACTTAAACATATGAACATGAAAATGTGATTGGTCTGTGGACGTTTTTACACCTGCA 645
 Y R E L R L L K H M K H E N V I G L L D V F T P A 93
 AGGTCTCTGGAGGAATTCATGATGTGTATCTGGTGACCCATCTCATGGGGCGAGAICGAACAACATIGIGAAA 720
 R S L E E F N D V Y L V T H L M G A D L N N I V K 118
 TGTCAGAAGCTTACAGATGACCATGTTTCAGTTCCTTATCTACCAAATTCCTCGAGGCTCTAAAGTATATACATTCA 795
C Q K L T D D H V Q F L I Y Q I L R G L K Y I H S 143
 GCTGACATAATTCACAGGGACCTAAACCTAGTAATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGAT 870
 A D I I H R D L K P S N L A V N E D C E L K I L D 168
 TTTGGACTGGCTCGGCACACAGATGATGAATGACAGGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATC 945
 F G L A R H T D D E M T G Y V A T R W Y R A P E I 193

FIG.16A

ATGCTGAAGTGGATGATTACACAGACAGTTGATATTGGTCAGTGGGATGCATAATGGCCGAGCTGTTGACT 1020
 M L N W M H Y N Q T V D I W S V G C I M A E L L T 218
 GGAAGAACATTGTTTCCTGGTACAGACCATATTAAACAGCTTCAGCAGATTATGCGTCTGACAGGAACACCCCCC 1095
 G R T L F P G T D H I N Q L Q Q I M R L T T G T P P 243
 GCTTATCTCATTACAGGATGCCAAGCCATGAGGCAAGAACTATATTCAGTCTTTGACTCAGATGCCGAAGATG 1170
 A Y L I N R M P S H E A R N Y I Q S L T Q M P K M 268
 AACTTTGCGAATGTATTTATTGGTGCCCAATCCCTGGCTGTGACITGCTGGAGAAGATGCTTGTATTGGACTCA 1245
 N F A N V F I G A N P L A V D L L E K M L V L D S 293
 GATAAGAGAATTACAGCGGCCCAAGCCCTTGCACATGCCCTACCTTTCAGTACAGGATCCTGATGATGAACCA 1320
 D K R I I A A Q A L A H A Y F A Q Y H D P D E P 318
 GTGGCCGATCCTTATGATCAGTCCTTTGAAAGCAGGGACCTCCTTATAGATGAGTGGAAAGCCTGACCTATGAT 1395
 V A D P Y D Q S F E S R A D L L I D E W K S L T Y D 343
 GAAGTCATCAGCTTTGTGCGCCACCACCCCTTGACCAAGAAGAGATGGAGTCTTGAGCACCT...2330nts...
 E V I S F V P P P P L D Q E E M E S

FIG.16B

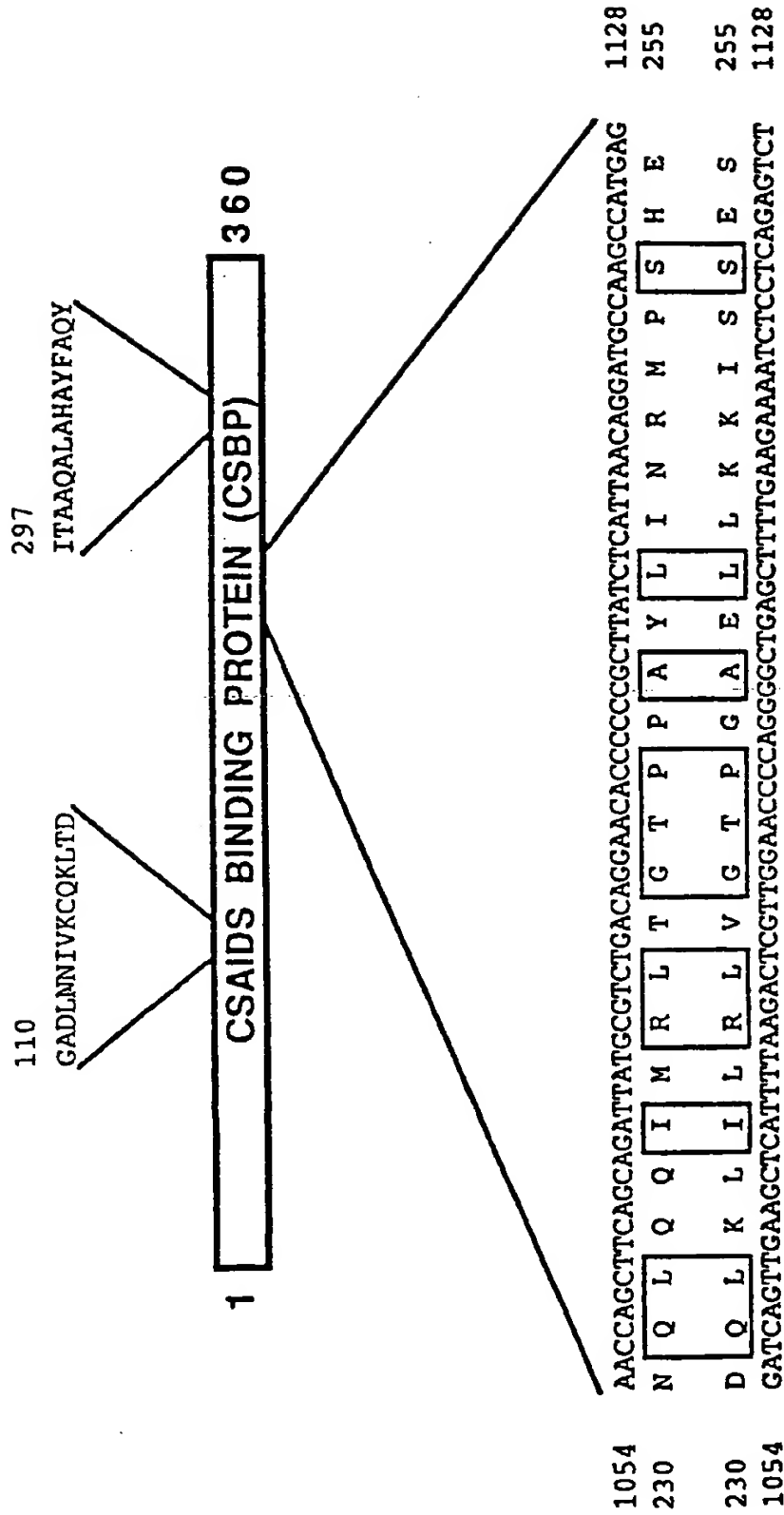
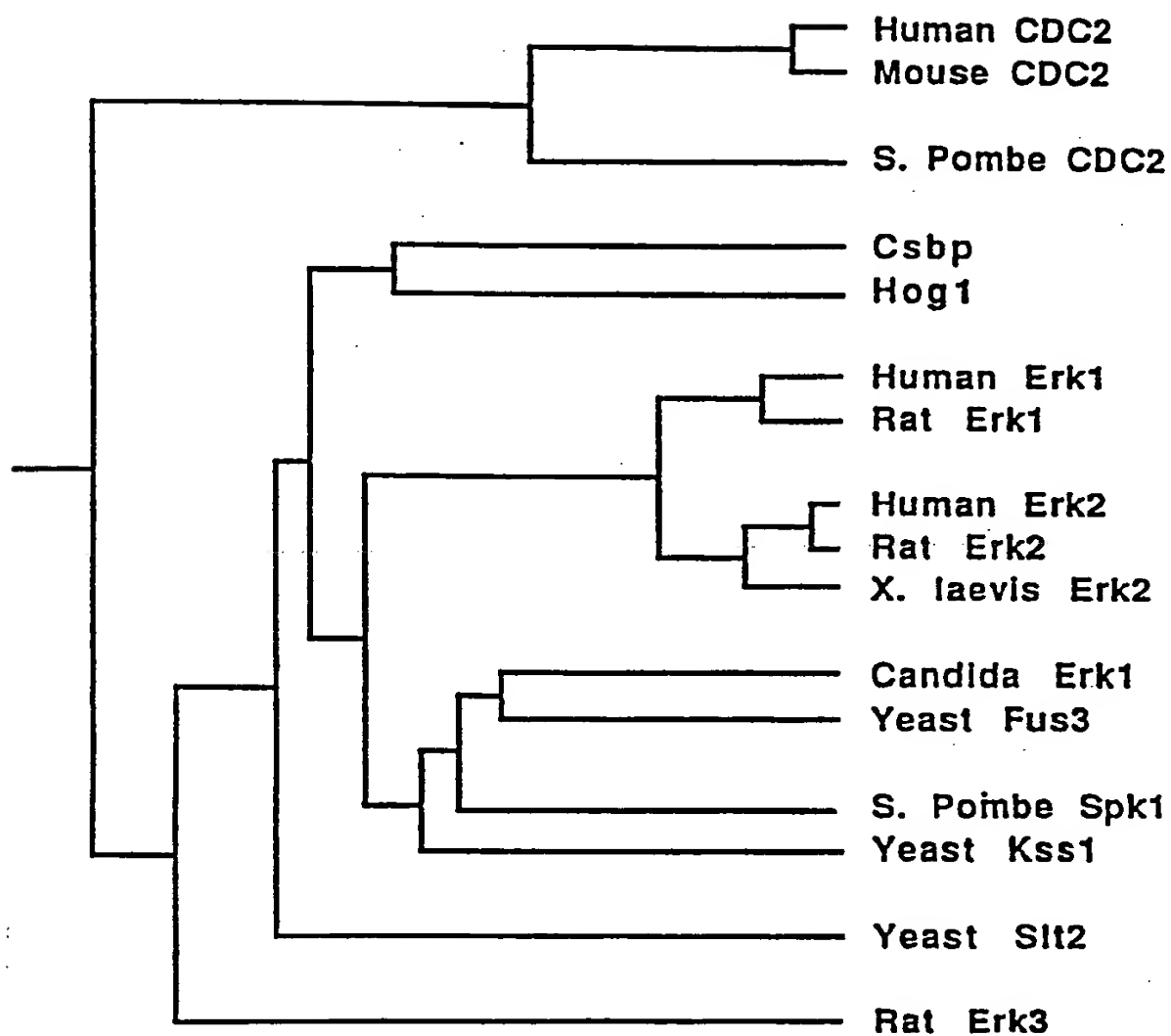


FIG.17

**FIG.18**

Human Erk1	1	MAAAAAAGGG	GGEPRRTEGV	GP GVPGEVEM	VKGQ....PF	DVGPRYTQLQ	50
Human Erk2		MAAAAAAGAG	P.....EM	VRGQ....VF	DVGPRYTQLQ	
Csbp	MSQERPTF	YRQELNKTIV	EVERYONIS	
Yeast Hog1	MTTNEEF	IRTQIFGTVF	EITNRYNDIN	
Identity	RY...L.	
Human Erk1	51	YIGEGAYGMV	SSAYDHVRKT	RVAIKKIS.P	FEHQTYCQRT	LREIQILRF	100
Human Erk2		YIGEGAYGMV	CSAYDNVNKV	RVAIKKIS.P	FEHQTYCQRT	LREIKILRF	
Csbp		PVGSGAYGSV	CAAFDTKTGL	RVAVKKLSRP	EQSIIHAKRT	YRELRLKHM	
Yeast Hog1		PVGMGAFGLV	CSATDTLTSQ	PVAIKKIMKP	ESTAVLAKRT	YRELKLKHL	
Identity		..G.GA.G.V	..A.D.....	..VA.KK...P	F.....RT	..RE...L...	
Human Erk1	101	RHENVTGIRD	IL.RASTLEA	MRDVYIVQDI	METDIYKLLK	SQQLSNDHIC	150
Human Erk2		RHENVTGIND	II.RAPTIEQ	MKDVYIVQDI	METDIYKLLK	TQHLSDNHIC	
Csbp		KHENVTGLLD	VFTPARSLER	FNDVLYTHE	MGADLNNIVK	CQKLTDDHVQ	
Yeast Hog1		RHENVTCLQD	IF.....LSP	LEDIYFVTEL	QGTDLHRLLO	TRPLEKQFVQ	
Identity		..HEN..I....DD.Y.V...L	...DL.....	...L.....	
Human Erk1	151	YFLYQILRGL	KYIHSANVLH	RDLKPSNLLI	NTTCDEKICD	FGLARADPE	200
Human Erk2		YFLYQILRGL	KYIHSANVLH	RDLKPSNLLI	NTTCDEKICD	FGLARVADPD	
Csbp		FLIYQILRGL	KYIHSADITH	RDLKPSNLAV	NEDCEKICLD	FGLARHTDDE	
Yeast Hog1		YFLYQILRGL	KYVHSAGVIH	RDLKPSNILI	NENDEKICD	FGLARIDDPQ	
Identity		...YQILRGL	KY.HSA...H	RDLKPSN...	N...C.LKI.D	FGLAR..D..	
Human Erk1	201	HDHTGFLTEY	VATRWYRAPE	IMNSKGYTK	SIDTWSVGC	LAEMLSNRPI	250
Human Erk2		HDHTGFLTEY	VATRWYRAPE	IMNSKGYTK	SIDTWSVGC	LAEMLSNRPI	
Csbp	MTGY	VATRWYRAPE	IMNWMHYNQ	TVDTWSVGC	MAELLTGRTL	
Yeast Hog1	MTGY	VSTRYRAPE	IMLTWQKYDV	EVDTWSAGCI	FAEMIEGKPL	
Identity	T..	V.TR.YRAPE	IML....Y..	..DIWS.GCI	..AE.....	
Human Erk1	251	FPGKHYLDQL	NHILGILGSP	SQEDLNCIIN	MKARNYLOS	PSKTKVAAWAK	300
Human Erk2		FPGKHYLDQL	NHILGILGSP	SQEDLNCIIN	LKARNYLLS	PHKNKVPWNR	
Csbp		FPGTDHINQL	QQIMRLTGTP	PAYLINRMPS	HEARNYIQSI	TQMPKMFAN	
Csbp2		-----DQL	KLTLRLVGTP	GAELLKKISS	ES-----		
Yeast Hog1		FPGKDHVHQF	SIITDLLGSP	PKDVINTICS	ENTLKFVTS	PHRDPPIFSE	
Identify		FPG.....Q.	..I.....G.PSL	
Human Erk1	301	LEPKSDSKAL	DLLDRMLTFN	PNKRITVEEA	LAHPYLEQY	DPDDEPVAEE	350
Human Erk2		LEPNADSKAL	DLLDKMLTFN	PHKRLEVEQA	LAHPYLEQY	DPDDEPIAEA	
Csbp		VEIGANPLAV	DLEKMLVLD	SDKRITAAQA	LAHAYFAQYH	DPDDEPVADP	
Yeast Hog1		REKTVEPDVAV	DLEKMLVFD	PKKRITAAQA	LAHPYSAPYH	DPDDEPVADA	
Identity		..F.....A.	DLL..ML...	..KRI....A	LAH.Y...Y.	DP.DEP.A..	
Human Erk1	351	PFTFAMELDD	EPKERLKELI	FQETARFQPG	VLEAP.....	400
Human Erk2		PFKFDMELDD	EPKEKLKELI	FEETARFQPG	YRS.....	
Csbp		.YDQSFESRD	ELIDEWKSLT	YDEVISEVPP	PLDQEEEMES	
Yeast Hog1		KFDWHFNDAD	LPVDTWRVMM	YSILDEHKI	GGSDGQIDIS	ATFDDQVAAA	
Identity	D	L.....	..E...F...	

FIG.19

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MW AA Promoter



enterokinase
cleavage site

Met Asp Tyr Lys Asp Asp Asp Lys

M1 antibody
binding site

FLAG peptide



FIG.20


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1  GGAACCCGCA CCACTGGAGCCTTAGCGGGCGCAGCAGCTGGAAACGGGAGTACTGCGACGACCCCGAGTCCGGCC
76  TTGTAGGGGGGAAGGTGCAGGGAGATCGCGGGCGGCGAGTCTTGAGCGCCGGAGCGCGTCCCTGCCCTTAGCGG
151  GGCTTGCCCCAGTCGCAGGGGCACATCCAGCCGCTGCGGCTGACAGCAGCCGCGCGCGGGAGTCTGCGGGGTC
226  GCGGCAGCCGCACCTGCGCGGGCGGACCAAGGTCCCGCCCGCGCTGGCGGGCGCAGCAAGGCGCGGGAGAG
301  GGTGCGGGTGCAAGCGGGGGCCCCACAGGGCCACCTTCTTGCCCCGGCGCTGCCGTAATAATGTC TCAGGAGA
    -19      MetSerGlnGluA
376  GGCCACGTTCTACCGGCAGGAGCTGAACAAGACAAATCTGGGAGGTGCCCGAGCGTTACCAGAAACCTGTCTCCAG
    6  rgProThrPheTyrArgGlnGluLeuAsnLysThrIleTrpGluValProGluArgTyrGlnAsnLeuSerPro
451  TGGGCTCTGGCGCCCTATGGCTCTGTGTGTCTGCTGCTTTTGACACAAAAACGGGTTACGTGTGGCAGTGAAGAAGC
31  alGlySerGlyAlaTyrGlySerValCysAlaAlaPheAspThrLysThrGlyLeuArgValAlaValLysLysL
526  TCTCCAGACCATTTTCAGTCCATCATTCATGCGAAAAGAACCTACAGAGAACTGCGGTTACTTAAACATATGAAAC
56  euSerArgProPheGlnSerIleIleHisAlaLysArgThrTyrArgGluLeuArgLeuLysHisMetLysH

```

FIG. 21A

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601 ATGAAAAATGTCATTCGGTCTGTTGGACGTTTTTACACCTGCAAGGTCTCTGGAGGAAATTCAATGATGTGTATCTGG
81  isGluAsnValIleGlyLeuLeuAspValPheThrProAlaArgSerLeuGluGluPheAsnAspValTyrLeuV

676 TGACCCATCTCATCGGGGCAGATCTGAACAACATTTGTGAAATGTCAGAAAGCTTACAGATGACCATGTTTCAGTTCC
106 alThrHisLeuMetGlyAlaAspLeuAsnAsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheL

751 TTATCTACCAAAATTCTCCGAGGTCTAAAGTATATACATTACGCTGACATAATTACACGGGACCTAAAACCTAGTA
131 euIleTyrGlnIleLeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSera

826 ATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTCGATTTTGGACTGGCTCGGCACACAGATGATGAAATGA
156 snLeuAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHisThrAspAspGluMetT

901 CAGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATGCTGAACCTGGATGCAATTAACACAGACAGTTG
181 hrGlyTyrValAlaThrArgTrpTyrArgAlaProGluIleMetLeuAsnTrpMetHisTyrAsnGlnThrValA

976 ATATTTGGTCAGTGGATGCATAATGGCCGAGCTGTTGACTGGAAGAACATTGTTTCTGCTGACAGACCATATTA
206 spIleTrpSerValGlyCysIleMetAlaGluLeuLeuThrGlyArgThrLeuPheProGlyThrAspHisIleA

1051 AccagcttcagcagATTAtgcgtctgacaggaaacACCCCCcgcttatctcattaacaggatgccaagccatgagG
231 snGlnLeuGlnGlnIleMetArgLeuThrGlyThrProProAlaTyrLeuIleAsnArgMetProSerHisGluA

1126 CAAGAAACTATATTTCAGTCTTTGACTCAGATGCCGAAGATGAACCTTTGCCGAATGTAATTTATTTGGTGCCAATCCCC
256 laArgAsnTyrIleGlnSerLeuThrGlnMetProLysMetAsnPheAlaAsnValPheIleGlyAlaAsnProL

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FIG. 2IB

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SUBSTITUTE SHEET (RULE 26)

1201 TGCCTGTCGACTTGCTGGAGAAGATGCTTGTTATTGGACTCAGATAAGAGAAATTACAGCGGCCCAAGCCCTTGCAC
281 euAlaValAspLeuLeuGluLysMetLeuValLeuAspSerAspLysArgIleThrAlaAlaGlnAlaLeuAlaH
1276 ATGCCCTACTTTGCTCAGTACCACGATCCTGATGATGAACCCAGTGGCCGATCCTTATGATCAGTCCTTTTGAAAGCA
306 isAlaTyrPheAlaGlnTyrHisAspProAspAspGluProValAlaAspProTyrAspGlnSerPheGluSerA
1351 GGGACCTCCTTATAGATGAGTGGAAAAGCCCTACCTATGATGAAGTCATCAGCTTTGTGCCACCACCCCTTGACC
331 rgAspLeuLeuIleAspGluTrpLysSerLeuThrTyrAspGluValIleSerPheValProProLeuAspG
1426 AAGAAAGAGATGGAGTCCTGAGCACCTGGTTTCTGTTCTGTGATCCCACCTTCACTGTGAGGGGAAGGCCCTTTTCA
356 InGluGluMetGluSerEnd
1501 CGGGAACTCTCCAAATATTATTCAAGTGCCCTCTGTGTCAGAGATTTCCCTCCATGGTGAAGGGGGTGTGCGTGC
1576 GTGTGCCGTGCGTGTAGTGCTGTGCAATGTGTGTCTGTCTTTGTGGGAGGGTAAGACAAATATGAACAAACTAT
1651 GATCACAGTGACTTTACAGGAGGTGTGCGATGCTCCAGGGCAGCCCTCCACCTTGCTCTTCTTCTGAGAGTTGGC
1726 TCAGGCAGACAGAGCTGCTGTCTCTTTTAGGAAATATGTTCAATGCAAGTAAAGTAAAAAATATGAATTGTCCCCAATC

FIG.21C

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SUBSTITUTE SHEET (RULE 26)

1801 CCGGTCA TGCTTTTGCCACTTTGGCTTCTCTGTGACCCCACTTGACGGTGGCGGTAGACTTIGACAAATCCC
1876 ACAGTGGCACGGAGAGAAAGGCCCATACCTTCTGGTTGCTTTCAGACCTGACACCGTCCCTCAGTGATACGTACAGC
1951 CAAAAGGACCACCTGGCTTCTGTGCACTAGCCCTGTGATTAACTTGTCTTAGTATGGTTCTCAGATCTTGACAGTA
2026 TATTTGAAACTGTAAATA TGTTTGTGCTTAAAGGAGAGAGAAAGTGTAGATAGTTAAAGACTGCAGCTGCT
2101 GAACTTCTGAGCCGGGCAAGTCGAGAGGGCTGTGGA CAGCTGCTTGTGGGCCCCGGAGTAATCAGGCAGCCTTCA
2176 TAGGGGTCA TGTTGTGCA TGAGGACATGCGTATATGTGCGTCTCTCTTTCTCCCTCACCCCCAGGTGTTGCCA
2251 TTTCTCTGCTTACCTTTCACCTTTGGTGAGAGGTTTCTTGAATATCTGCCCCAGTAGTCAGAAAGCAGGTTCTTG
2326 ATGTCATGTACTTCTCTGTACTCTTTATTCTAGCAGATGAGGATGTGTTTTIGCAGCTCTTIGCTATTTIGAGCA
2401 TGCACAGCTGCTGTCTGCTCTCTTCAGGAGGCCCTGGTGTGAGGAGGTTTGGCCAGTGAAGACTTCTTGGGTA
2476 GTTTAGATCCCATGTCACCTCAGCTGATATTATGGCAAGTATATCACCTCTCTTTCAGCCCCCTAGTGCTATTCTG
2551 TGTGAAACAAATTGATACTTCAGGTGCTTTTGTATGTGAAATCATGAAAGAGGAA CAGGTGGATGTATAGCAT
2626 TTTTATTTCATGCCATCTGTTTTCAACCAACTATTTTTGTAGGAATTATCATGGGAAAGACCCAGGGCTTTTCCCAG
2701 GAATATCCCAAACCTTCGGAAACAAGTTATTCTCTTCACTCCCAATAACTAATGCTAAGAAATGCTGAAAAATCAAA

FIG.2ID

2776 GTAAAAAATTAAAGCCCATAGGCCAGAAACTCCTTTTGGCTGTCTTTCTCTAAATATGATTACTTTTAAATAAAAA
2851 AAGTAACAAGGTGTCTTTTCCACTCCTATGGAAAAGGGTCTTCTTGGCAGCTTAAACATTGACTTCTTGGTTTGGG
2926 GAGAAAATAAATTTTGTTCAGAAATTTTGTATATTTGTAGGAATCCCCTTTGAGAATGTGATTCCTTTTGTATCGGAG
3001 AAAGGGCAAAATTATTTTAATAATTTTGTATTTTCAACTTTATAAAGATAAAATATCCTCAGGGGTGGAGAAGTGTCT
3076 GTTTTCATAAATTTTGTCTGAATTTTCAGGCAATTTTGTCTTACATGAGGACTCATATATTTAAGCCCTTTTGTATAATA
3151 GAAAGTATAAAGTCACTTCCAGTGTGGCTGTGTACAGAAATCTTGTATTTTGGGCCAAAGGTGTTTCCATTTCTCA
3226 ATCAGTCAGTGATACATGTACTCCAGAGGGACRGGGTGGACCCCTGAGTCAACTGGAGCAAGAAGGAAGGAGG
3301 CAGACTGATGGCGATTCCCTCTCACCCGGGACTCTCCCCCTTTTCAAGGAAAGTGAACCTTTTAAAGTAAAGGCCCTC
3376 ATCTCCCTTTATTTGCAGTTCAAAATCCTCACCATCCACAGCAAGATGAATTTTATCAGCCCATGTTTGGTTGTAAATG
3451 CTCGTGTGATTTCCCTACAGAAATACTGCTCTGAATATTTTGTATAAAGGTCTTTTGCACATGTGACCACATACGT
3526 GTTAGGAGGCTGCATGCTCTGGGAAGCCCTGGACTCTAAGCTGGAGCTCTTGGGAAGAGCTCTTCGGTTTCTGAGCAT
3601 AATGCTCCCATCTCCTGATTTTCTCTGAACAGAAAAACAAAAGAGAGAAATGAGGGAATTTGCTATTTTATTTGTATT
3676 CATGAACCTTGGCTGTAAATCAGTTATGCCGTATAGGATGTCAGACAAATACCACCTGGTTAAAAATAAAGCCCTATTTTT
3751 CAAATTTAAAAAATAAAAAAATAAAA 3775

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FIG.2IE

SUBSTITUTE SHEET (RULE 26)

1 CGCCCCAGTCGACGGGCACATCCAGCCCGCTGGCGGTGACAGCAGCCGCGCGGGAGTCTGCGGGGTGCGG
 76 CAGCCCGACCTGCGCGGGCGACACAGCGCAAGGTCCCGCCGCGTGGCGGGCAGCAAGGGCGGAGACGGGTG
 151 CGGGTGACGGCGGGGCCCCACAGGGCCACCTTCTTGCCCGCGGCTGCCGCTGGAATAATGTCTCAGGAGAGGCC
 -18 MetSerGlnGluArgPr
 226 CACGTTCTACCGGACGAGCTGAACAAGACAATCTGGGAGGTGCCCGAGCGTTACCAGAACCTGTCTCCAGTGGG
 7 oThrPheTyrArgGlnGluLeuAsnLysThrIleTrpGluValProGluArgTyrGlnAsnLeuSerProValGln
 301 CTC'TGGCGCCTATCGCTCTGTGTGTCTGCTTGTGACACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGCTCTC
 32 ySerGlyAlaTyrGlySerValCysAlaAlaPheAspThrLysThrGlyLeuArgValAlaValLysLysLeuSe
 376 CAGACCATTTCAGTCCATCATTCATCGGAAAAAGAACCTACAGAGAACTCCGGTTACTTAAACATATGAAACATGA
 57 rArgProPheGlnSerIleIleHisAlaLysArgThrTyrArgGluLeuArgLeuLysHisMetLysHisGln
 451 AAATGTGATTGGTCTGTGTGGACGTTTTTACACCTGCAAGGTCTCTCGGAGGAATTCAAATGATGTGTATCTCGTGAC
 82 uAsnValIleGlyLeuLeuAspValPheThrProAlaArgSerLeuGluGluPheAsnAspValTyrLeuValTh
 526 CCATCTCATGGGGCAGATCTGAACAACATTGTGAAATGTCAAGCTTACAGATGACCATGTTTCAGTTCCTTAT
 107 rHisLeuMetGlyAlaAspLeuAsnAsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheLeuIle
 601 CTACCAAATTCTCCGAGGTCTAAAGTATATACATTACAGCTGACATAATTCACAGGGACCTAAACCTAGTAATCT
 132 eTyrGlnIleLeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSerAsnLe
 676 AGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGATTTTGGACTGGCTCGGCACACAGATGATGAATGACAGG
 157 uAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHisThrAspAspGluMetThrGln

FIG.22A

751 CTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATGCTGAACCTGGATGCAATTACAACACAGACAGTTGATAT
 182 yTyrValAlaThrArgTrpTyrArgAlaProGluIleMetLeuAsnTrpMetHisTyrAsnGlnThrValAspIle

 826 TTGGTCAGTGGGATGCATAATGGCCGAGCTGTGACTGGAAGAACAATTGTTTCTGCTGACAGCCATATTGATCA
 207 eTrpSerValGlyCysIleMetAlaGluLeuLeuThrGlyArgThrLeuPheProGlyThrAspHisIleAspGly

 901 GTTGAAGCTCAATTTAAGACTCGTTGGAACCCACGGGCTGAGCTTTTGAAGAAATACTCCTCAGAGTCTGCAAG
 232 nLeuLysLeuIleLeuArgLeuValGlyThrProGlyAlaGluLeuLysLysIleSerSerGluSerAlaAr

 976 AACTATATTTCAGTCTTTGACTCAGATGCCGAGATGAACCTTTCGGAATGTATTTATTGGTGCCCAATCCCCCTGGC
 257 gAsnTyrIleGlnSerLeuThrGlnMetProLysMetAsnPheAlaAsnValPheIleGlyAlaAsnProLeuAl

 1051 TGTCGACTTGCTGGAGAAAGATGCTTGTATTGGACTCAGATAAGAGAAATTACAGCGGCCCAAGCCCTTGCACATGC
 282 aValAspLeuLeuGluLysMetLeuValLeuAspSerAspLysArgIleThrAlaAlaGlnAlaLeuAlaHisAl

 1126 CTACTTTGCTCAGTACCACGATCCTGTATGATGAACACAGTGGCCGATCCTTATGATCAGTCCCTTTGAAAGCAGCGGA
 307 aTyrPheAlaGlnTyrHisAspProAspAspGluProValAlaAspProTyrAspGlnSerPheGluSerArgAs

 1201 CCTCCTTATAGATGAGTGGAAAAGCCTGACCTATGATGAAGTCATCAGCTTTGTGCCACCCACCCCTTGACCAAGA
 332 pLeuLeuIleAspGluTrpLysSerLeuThrTyrAspGluValIleSerPheValProProProLeuAspGlnGly

 1276 AGAGATGGAGTCTCTGAGCACCTGGTTTCTGTTCTGTGATCCCACTTCACCTGTGAGGGGAAGGCCTTTTTCACGGG
 357 uGluMetGluSerEnd

 1351 AACTCTCCAAATATTATTCAAGTGCCCAAAA 1381

FIG.22B

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) :C07H 21/00, 21/04; C07K 14/435; C12N 15/00; C12Q 1/00 US CL :536/23.5; 530/350; 435/320.1; 252.3, 69.1, 7.2; 436/503, 504 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 536/23.5; 530/350; 435/320.1, 252.3, 69.1, 7.2; 436/503, 504 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, DIALOG - BIOTECH FILES, GENEMBL SEQUENCE DATABASES		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US, A, 5,317,019 (BENDER ET AL.) 31 May 1994, see entire document.	1-20
A	US, A, 4,794,114 (BENDER ET AL.) 27 December 1988, see entire document.	1-20
A	US, A, 4,780,470 (BENDER ET AL.) 25 October 1988, see entire document.	1-20
A	US, A, 4,778,806 (BENDER ET AL.) 18 October 1988, see entire document.	1-20
A	Agents and Actions, Volume 27, No. 3/4, issued 1989, J. C. Lee et al, "Effect of SK & F 86002 on cytokine production by human monocytes", pages 277-279, see entire document.	1-20
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"Z" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 09 DECEMBER 1994	Date of mailing of the international search report 09 JAN 1995	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIZABETH C. KEMMERER <i>E. Kemmerer</i> Telephone No. (703) 308-0196	

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-20, drawn to nucleic acids encoding a CSBP, the protein, vectors, host cells, a method of expressing the nucleic acids, and a method of identifying a CSAID using the protein.
- II. Claims 21-23, drawn to a method of identifying CSBP ligands.
- III. Claims 24 and 25, drawn to antagonists and agonists of CSBP, and pharmaceutical compositions comprising same.
- IV. Claim 26, drawn to antisense molecules.
- V. Claims 27 and 28, drawn to antibodies.
- VI. Claim 29, drawn to a transgenic animal.
- VII. Claim 30, drawn to a method of screening compounds using a fusion protein.

The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Beginning with Invention II, each subsequent invention introduces a new concept not required by Invention I or any of the other Inventions. For example, Invention II introduces the concept of ligands and natural sources thereof, Invention III introduces antagonists/agonists and chemical sources thereof, Invention IV introduces antisense technology which is useful in gene expression modulation and nucleic acid probing techniques, Invention V introduces antibodies and the entire field of immunology, Invention VI introduces transgenic technology, and Invention VII introduces the field of fusion protein engineering.